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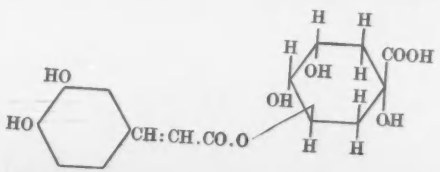
The isolation of chlorogenic acid from the apple fruit

By A. C. HULME

Volume 53 (1953), No. 3

p. 337, col. 1, formula for chlorogenic acid to read:

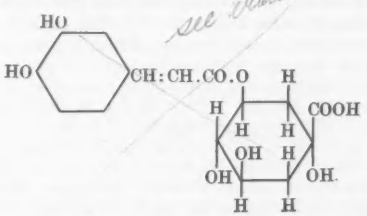


# The Isolation of Chlorogenic Acid from the Apple Fruit

By A. C. HULME
*Ditton Laboratory, Department of Scientific and Industrial Research, East Malling, Maidstone, Kent*

(*Received* 10 *July* 1952)

Chlorogenic acid, the depside formed by the condensation of caffeic and quinic acids, was first obtained in crystalline form by Gorter (1909) from green coffee beans, although considerably earlier Ludwig & Kromeyer (1854) had noted the presence in the seeds of the sunflower of a compound which was undoubtedly chlorogenic acid. The acid was first thought to be a glycoside of caffeic acid, but Gorter (1909) showed that the 'sugar' was, in fact, quinic acid and that the acidity was due to the free carboxyl group of this acid. Finally, Fischer (see Freudenberg, 1933) proved chlorogenic acid to have the following structure:




Gorter found the acid not only in coffee beans but also in coffee leaves, latex of *Castilloa elastica*, and the seeds of *Helianthus annuus*, *Kopsia flavida* and *Strychnos nux vomica*. On the basis of a colour reaction involving ferric chloride, he suggested that the acid was present in the plants of a large number of natural orders but, as Onslow (1931) has pointed out, Gorter's test will give a positive result with any *o*-dihydroxyphenolic compound. Oparin (1921, 1927) suggested that chlorogenic acid might act as a 'respiration-pigment' as defined by Palladin (1912) and claimed that it could bring about the oxidation of amino-acids and peptides especially in the presence of phosphates.

Recent interest in chlorogenic acid has centred on its function as a substrate for polyphenolase and it appears that the darkening of fruits on injury may be due to the oxidation of chlorogenic acid rather than to the oxidation of true tannins (Joslyn & Ponting, 1951). Cruess & Alsberg (1934) consider that a glucoside of caffeic acid and phenol obtained from olives is responsible for the browning of these fruits. Rudkin & Nelson (1947) showed that the addition of small quantities of chlorogenic acid, which they isolated from the sweet potato, increased the oxygen uptake of, and also the carbon dioxide given off by, thin slices of the root of this plant.

The isolation of considerable quantities of quinic acid from the young fruits of Worcester Pearmain apples, and also its presence in pears and other fruits, suggested (Hulme, 1951) that one of several possible functions of the acid might be to combine with caffeic acid to form chlorogenic acid. The reactions of extracts of the young apples suggested that, in fact, they might well contain chlorogenic acid since they gave a positive Gorter test, slowly turned brown on the surface on being made alkaline, and gave paper chromatograms showing a spot with an intense ultraviolet fluorescence turning duck-egg green on exposure to the fumes of ammonia. Meanwhile, the presence of chlorogenic acid has been established in the vegetative parts of apple and pear trees and in the juice of mature pears (Bradfield, Flood, Hulme & Williams, 1952).

In experiments designed to isolate amino-acids from extracts of 50–100 kg. of the tissue of young apples, by absorption on large columns of cation-exchange resin followed by absorption of organic acids on anion-exchange resins, there was found to be present in the extracts a substance (or substances) which, when in contact with the anion-exchange resin or with the ammonia used to displace the amino-acids from the cation-exchange resin, was apparently transformed into a dark-coloured compound which clogged the columns. The apple extracts appeared to contain a considerable amount of this substance, and from its behaviour on the columns it seemed likely that absorption on the cation-exchange resin was not ionic but due to van der Waal's forces, and that the strongly basic nature of the anion-exchange resin (Dowex 2, R. W. Greef & Co. Ltd.) was modifying the substance to give the strongly pigmented compound. The substance behaved, in fact, like a soluble *o*-dihydroxyphenolic compound and its removal from the apple extracts was essential to a satisfactory separation of the amino-acids and organic acids.

For the removal of aromatic amino-acids, which behave in an anomalous manner on cation-exchange resins (Partridge, 1949), partially deactivated charcoal prepared by the method of Schramm & Primosigh (1943) has been used. It was found that

if the apple extracts were treated with sufficient of this charcoal to remove all colour, the extracts no longer darkened on being made alkaline. Furthermore, the substance originally responsible for the alkali-darkening could be recovered from the charcoal by displacement with the phenol-acetic acid reagent of Schramm & Primosigh (1943). As described below, considerable quantities of this substance, amounting to several grams, were isolated, purified and identified as chlorogenic acid.

## EXPERIMENTAL

Several thousand immature fruits of average weight 13·6 g. were gathered from Worcester Pearmain apple trees and placed in galvanized-iron tanks through which was passed a stream of humidified air at 15°. After 7 days the fruits were free of starch (the removal of starch was required for work not reported here on the ethanol-insoluble material of the fruit). After rapidly halving the fruits (at 1°) and removing the embryonic seeds they were frozen at −20°. When frozen, the fruit was peeled at −10° and the pulp tissue ground to a fine powder, also at −10°. Approximately 7 kg. of this powder were plunged, in batches of 500 g., into boiling 95% ethanol (the final ethanol concentration was ~80%). The combined batches (liquid and tissue) were poured into large Soxhlet thimbles. All the thimbles were placed in a Soxhlet type extractor and extracted under reduced pressure with 80–85% ethanol until the liquid leaving the thimbles was colourless (48 hr.). All the ethanol extracts were combined (approximately 14 l.) and the ethanol removed below 45°. The residual liquid (2 l.) was filtered through a pad of asbestos to remove green, waxy material and the reddish brown, slightly opalescent filtrate was treated with a few ml. $CHCl_3$ and stored at 1°. Nine similar extractions of 7 kg. of pulp tissue were carried out, approximately 2 l. of filtered extract being obtained from each. Each 2 l. batch was shaken for several hours with 80–90 g. of acid-treated charcoal (Schramm & Primosigh, 1943; Partridge, 1949). The charcoal was filtered off and washed until free of sugar. The various charcoal filter cakes were stirred into a solution of 20% (v/v) acetic acid solution containing phenol (5%, w/v) (Partridge, 1949)—about 8 l. in all being used—and stored at 1° for several days. Subsequent operations were carried out on batches of approx. 1 l. of the well stirred charcoal suspension. Each batch was filtered, the charcoal washed with a little dilute acetic acid and the filtrate and washings shaken with 1 l. of ethyl ether in 100 ml. lots in a separating funnel to remove the phenol. The acetic acid in the phenol-free residual liquid, which was orange coloured, was removed by repeated evaporation and dilution with water. The final evaporate, about 500 ml., had a pH of between 3·2 and 3·3. It was rapidly neutralized (pH 6·8) by the addition of solid $Na_2CO_3$; during this stage it was impossible to prevent some darkening of the liquid. Neutral lead acetate (5 ml., 0·5 M) was added and the small grey precipitate which formed was discarded since it contained a large amount of impurity in relation to its chlorogenic acid content. The deep-red supernatant liquid was treated with excess neutral lead acetate; the precipitate formed was dissolved in 5% acetic acid and reprecipitated at pH 8·5; this precipitate was suspended in water, the lead carefully removed with $H_2SO_4$, the lead-free filtrate brought to pH 2·0 and extracted with ethyl acetate (1300 ml. in 100 ml. lots in a separating funnel, 'first extract', and then in a liquid-liquid extractor, 'second extract'), etc. following the procedure used by Rudkin & Nelson (1947) to isolate chlorogenic acid from sweet potato. Before extracting chlorogenic acid in the undissociated form with ethyl acetate, Rudkin & Nelson first made their extracts 2 M in $KH_2PO_4$ and then acidified them to pH 2. This addition of phosphate was found, in the present work, to cause the formation of orange-coloured, insoluble agglomerates during the extraction with ethyl acetate, and the crude chlorogenic acid obtained proved difficult to purify. The addition of phosphate was, therefore, discontinued after treatment of the first litre of charcoal eluate.

It was found advantageous to carry out all evaporations under reduced pressure in a stream of $N_2$.

The crude preparations (hereafter called 'crude chlorogenic acid preparations'), obtained by precipitation from the concentrated ethyl acetate extracts by the addition of excess light petroleum (b.p. 60–80°) followed by centrifugation, were dried *in vacuo* after treatment with a few drops of benzene. Unlike Rudkin & Nelson's crude preparations from the sweet potato they were coloured pink, the depth of colour increasing during drying. From the evidence presented below (under 'Examination and characterization of chlorogenic acid preparations from the apple'), and from microchemical tests, it appeared that these crude preparations contained, in addition to chlorogenic acid, various catechins. Attempts were made to purify the preparations by the counter-current distribution method as used by Rudkin & Nelson, but it was clear that the contaminants were not of the type of component A (closely related to chlorogenic acid) of these workers. Preliminary experiments using silica-gel chromatography also failed to effect a separation from all the coloured impurities. Since the catechins do not crystallize readily from water and, as Kirby, Knowles & White (1951) have shown, exhibit mutual solubilities, it was decided to try to crystallize the chlorogenic acid from water at 1° in presence of a little HCl (Freudenberg, 1933, mentions that mineral acids reduce the solubility of chlorogenic acid). This method proved immediately successful and after recrystallizing several times, washing the crystals each time with ice-cold water, and drying *in vacuo* over $P_2O_5$ a white crystalline compound was obtained which, on examination as described below, proved to be chlorogenic acid. To prevent the compound becoming slightly tinged with pink during drying it was necessary to place the wet crystals immediately in the desiccator (at 1°) and fill with $N_2$ before connecting to the vacuum pump.

### *Examination and characterization of chlorogenic acid preparations from the apple*

The author is greatly indebted to Dr J. M. Nelson of Columbia University for an authentic sample of chlorogenic acid which was compared with the material from the apple in the following procedures.

*Filter-paper chromatography.* Chromatograms were prepared on Whatman no. 2 'chromatographic' paper. The solvent system used was Campbell, Work & Mellanby's (1951) modification of Partridge's (1948) original 4:5:1 butanol/water/acetic acid mixture. Although the former authors inferred that

the upper layer of this mixture as prepared by them had the same composition as the upper layer of Partridge's system, it was found that phenolic bodies such as chlorogenic acid gave considerably elongated spots with the Partridge mixture, whereas they gave almost round spots with the Campbell, Work & Mellanby mixture (48 hr. allowed for esterification before use with both solvent systems). Paper chromatography showed that at least six substances giving an orange coloration when sprayed with the diazotized *p*-nitroaniline reagent of Bradfield, Penney & Wright (1947) (modified for use as a spray by the addition of sodium acetate, Bradfield (1948)) were present in the neutral lead acetate precipitate, and five of these continued to be present up to and including the crude light petroleum precipitate. From chromatographic evidence two of these compounds appeared to be epicatechin and (±)-catechin. No compounds reacting to the diazotized *p*-nitroaniline spray travelled faster than (±)-catechin. This rules out the presence of the epicatechin gallates which have considerably higher $R_F$ values than (±)-catechin (Bradfield & Bate-Smith, 1950). Filter-paper chromatograms of the recrystallized compound showed one spot only, fluorescing blue in ultraviolet light, changing to duck-egg green when exposed to the fumes of ammonia and giving a positive reaction when sprayed with Bradfield's modified diazo reagent (see above), or with Roberts's (1951) modification for spraying of Hoepsner's (1932) nitrite test, or with Partridge's (1948) ammoniacal silver nitrate reagent. Nelson's sample (see above) of chlorogenic acid, when run on chromatograms alongside the present preparation, gave identical results and an identical $R_F$ value (0·57).

*Chemical methods.* The crystalline compound had m.p. 207–209° not depressed by admixture with authentic chlorogenic acid (m.p. 207–209° corr.). (Found: C, 54·1; H, 5·4. Calc. for $C_{16}H_{18}O_9$: C, 54·24; H, 5·12%.) A sample was hydrolysed for 15 min. at 20° with potassium hydroxide (13%, w/v) and neutralized (pH 4) with sulphuric acid (10%, w/v) (cf. Freudenberg, 1933). The product contained chlorogenic acid, quinic acid and caffeic acid (chromatographic comparison with authentic specimens).

*Absorption spectra.* The absorption spectrum in ultraviolet light of the crude light petroleum precipitate in ethanol showed very high absorption below 240 mμ. and gave peaks at 283 mμ. and approx. 328 mμ., the former being considerably more pronounced than the latter. Bradfield & Penney (1948) showed that the maxima for the catechins, gallocatechins and catechin gallates (in ethanol) all occur between 271 and 280 mμ. In addition, all their catechins showed strong absorption below 240 mμ. These facts provide support for the suggestion arising from the paper-chromatographic data that the crude chlorogenic acid preparations were contaminated with a series of catechins.

The recrystallized chlorogenic acid preparations gave an ultraviolet absorption spectrum identical with that given by Nelson's sample of the acid (Fig. 1). The molar extinction coefficient at the peak wavelength of 328–300 mμ. ($\epsilon_{max.}$) was 19 700.

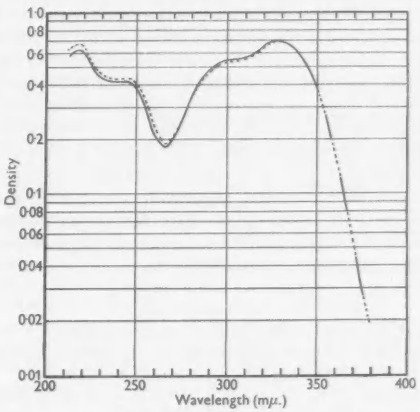



Fig. 1. Absorption spectra of chlorogenic acid obtained from the apple (Hulme), —; and from the sweet potato (Nelson), - - - -.

## DISCUSSION

These results demonstrate the presence of chlorogenic acid in the young fruits of the Worcester Pearmain apple. The 70 kg. of pulp tissue yielded approx. 19 g. of 'crude chlorogenic acid preparation' in the combined first ethyl acetate extracts and approx. 3·5 g. in the combined second ethyl acetate extracts. The light absorption measurements showed that the first ethyl acetate extracts were richer in chlorogenic acid in relation to 'catechins' than the second ethyl acetate extracts, so that some form of counter-current extraction should, in future work, greatly facilitate the separation of chlorogenic acid and catechin.

Approximately 3 g. of pure chlorogenic acid were obtained from the 19 g. of crude chlorogenic acid preparation from the first ethyl acetate extracts; practically none crystallized from the crude preparation from the second ethyl acetate extracts. Some of the acid undoubtedly remained uncrystallized from the catechin-rich supernatant liquids, as was confirmed by an examination of these liquids by filter-paper chromatography. Since catechin gallates, the only catechin compounds having a high molecular extinction coefficient between 270 and 280 mμ. (Bradfield *et al.* 1947; Bradfield & Penney, 1948) appear to be absent from

the crude chlorogenic preparations (see p. 338) it was possible, from an examination of the ultraviolet absorption spectra of these crude preparations, to conclude that at least twice as much chlorogenic acid was present in the 70 kg. of apple pulp tissue as was isolated in a crystalline form, i.e. 6–7 g. in all or approximately 10 mg./100 g. of pulp tissue. The same pulp tissue contains 800 mg./100 g. of quinic acid (Hulme, 1952) and, in spite of the disparity in these quantities, it is tempting to conclude that the two acids are metabolically related in the fruit. It is possible that one role of quinic acid is, by combining with the relatively insoluble caffeic acid, to render the *o*-dihydroxyphenolic properties of this latter acid more readily available in the form of the soluble chlorogenic acid. Chlorogenic acid would then be analogous to the glycosides of the insoluble flavone and flavonol pigments, the quinic acid residue in the former playing the same 'solubilizing' role as the sugar residues in the latter. Glycosides of caffeic acid are not commonly known to occur; the caffeic acid-phenol glycoside of olives of Cruess & Alsberg mentioned earlier appears to be the only one so far reported.

## SUMMARY

1. The occurrence and possible function of chlorogenic acid in plants is discussed.

2. A method is described whereby the acid can be isolated free of catechins from the young fruits of Worcester Pearmain apples.

3. An estimation is made of the amount of chlorogenic acid present in these young apples.

4. Its possible relationship to quinic acid, also present in the fruits, is discussed.

I wish to thank Dr A. E. Bradfield for valuable advice during the course of the work. Mr L. S. C. Wooltorton carried out much of the practical work involved in this paper.

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# Separation and Estimation of Blood Keto Acids by Paper Chromatography

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Owing to the interest attaching to the level of pyruvic acid in the blood, and its variations under physiological and pathological conditions, a number of methods have been devised for its estimation. Based on the earlier work of Dakin & Dudley (1913), the 'hydrazone' method was introduced by Barrenscheen & Dreguss (1931) and Case (1932). Its modifications by Peters & Thompson (1934), Lu (1939*a*), Bueding & Wortis (1940) and others, afforded a more specific method for the estimation of total keto acids than the 'bisulphite-binding' method of Clift & Cook (1932*a*). However, even the later precautions introduced by Friedemann & Haugen (1943), while improving still further the specificity of the method for the estimation of pyruvic acid, did not completely exclude interference from other keto acids present in blood or other biological materials.

The technique of paper chromatography (Consden, Gordon & Martin, 1944) seemed likely to be of particular value for the separation and identification of coloured compounds such as the 2:4-dinitrophenylhydrazones of keto acids, and Cavallini, Frontali & Toschi (1949*a*) have recently published a short account of the separation and estimation of these compounds by this technique. These workers later (Cavallini, Frontali & Toschi, 1949*b*) described the application of paper chromatography to the estimation of pyruvic acid and α-ketoglutaric acid in blood and urine. After extracting the coloured spots from the paper, colorimetric estimation in strongly alkaline solution yielded mean values of 0·28 mg. pyruvic acid and 0·21 mg. α-ketoglutaric acid/100 ml. blood in five samples from normal human subjects. Rossi & Di Leva (1951), using essentially the same method, found very much lower values for the pyruvic acid content of human blood, ranging from 0·06 to 0·13 mg./100 ml. for four normal subjects. α-Ketoglutaric acid was again found to be present in the blood extracts, and two further unidentified spots were also observed. LePage (1950) has separated the keto acids of normal and neoplastic rat tissues by chromatographic adsorption on columns of 'Hyflo Super-Cel', but he does not describe any application of this technique to human blood.

From a study of the blood 'pyruvate' levels, estimated by the Friedeman & Haugen (1943) technique, in a series of cases of polyneuritis (Joiner, McArdle & Thompson, 1950), we became interested in an exact identification of the raised levels of keto acids found in some of these patients in whom there was no reason to suspect a deficiency of vitamin $B_1$. In the case of a deficiency of this vitamin it is known that the raised level of blood keto acids is due largely to pyruvic acid, as this has been isolated and identified as its 2:4-dinitrophenylhydrazone (Thompson & Johnson, 1935; Johnson, 1936). In other types of polyneuritis, however, and particularly in those showing no biochemical response to massive parenteral thiamine therapy (i.e. no lowering of previously raised keto acid levels), it becomes important to identify the excess keto acid more precisely. We decided, therefore, to study the possibilities of paper-chromatographic techniques; in view of the low levels of pyruvic acid found in blood by the Italian workers, we began by re-investigating the procedure (methods of extraction, suitability of different solvents, etc.), outlined by them, comparing our chromatographic results with simultaneous estimations by the usual colorimetric technique.

In order to identify the coloured spots the 2:4-dinitrophenylhydrazones of a number of pure keto acids which might reasonably be expected to occur in blood were first prepared and studied.

## EXPERIMENTAL

Melting points are uncorrected. Micro-analyses were done by Weiler and Strauss, Oxford.

*General method for the preparation of 2:4-dinitrophenylhydrazones.* Excess of a saturated solution of 2:4-dinitrophenylhydrazine in 2N-HCl is added to an aqueous solution of the sodium salt of the keto acid, prepared by neutralizing 0·2 g. of the acid dissolved in 5 ml. water with the equivalent amount of $NaHCO_3$ dissolved in 5 ml. water. On standing at room temperature the 2:4-dinitrophenylhydrazones (referred to hereafter as 'hydrazones') crystallize out, and are purified by recrystallization. A further crop of crystals can be obtained by standing the separated mother liquor at 0–4° overnight.

*Pyruvic acid 2:4-dinitrophenylhydrazone.* Sodium pyruvate, prepared by the method of Robertson (1942), was used for the preparation of the hydrazone. This was recrystallized from glacial acetic acid: m.p. 218°. (Found: C, 40·2; H, 3·0; N, 20·5. Calc. for $C_9H_8O_8N_4$: C, 40·2; H, 3·0; N, 20·9%.)

*Oxaloacetic acid 2:4-dinitrophenylhydrazone.* The pure acid (m.p. 146°), prepared by standard methods, was used. The dry hydrazone was recrystallized from ethyl acetate in the cold by addition of light petroleum (b.p. 60–80°): m.p. 211°. (Found: N, 17·8. Calc. for $C_{10}H_8O_8N_4$: N, 17·9%.)

*α-Ketoglutaric acid 2:4-dinitrophenylhydrazone.* The pure acid (m.p. 110°) was prepared by the method of Schneider (1945); the hydrazone was recrystallized as for the oxaloacetic acid derivative: m.p. 219°. (Found: C, 40·8; H, 3·3; N, 17·0. Calc. for $C_{11}H_{10}O_8N_4$: C, 40·5; H, 3·1; N, 17·2%.)

*Acetoacetic acid 2:4-dinitrophenylhydrazone.* Acetoacetic acid was prepared as the sodium salt in aqueous solution by the method of Shaffer (1921). The hydrazone was prepared and recrystallized by the method of Clift & Cook (1932*b*), m.p. 124°. (Found: N, 20·3. Calc. for $C_{10}H_{10}O_6N_4$: N, 19·9%.)

*Acetone 2:4-dinitrophenylhydrazone.* This was prepared and recrystallized by standard methods: m.p. 126°.

*Preparation and extraction of keto acid hydrazones from blood.* Immediately after withdrawal from the body, 5–7 ml. blood are added to 20 ml. 5% (w/v) metaphosphoric acid in a weighed tube. It is important to collect the blood with the minimum of venous stasis, and no clenching and unclenching of the hand is allowed before collection. The tube is reweighed, allowed to stand for 10 min. at room temperature, and then centrifuged.

To 20 ml. of the centrifugate 2 ml. 0·2% (w/v) 2:4-dinitrophenylhydrazine in 2N-HCl are added, and the mixture incubated for 20 min. at 38°.

The mixture is then extracted in a separating funnel with 5 ml. ethyl acetate. The layers are separated and the aqueous phase re-extracted with 5 ml. ethyl acetate. The two ethyl acetate extracts, which are in the form of a fine emulsion, are centrifuged for 20 min. at 4000 rev./min. The clear, upper ethyl acetate layer is then removed with a teat-pipette, and the combined aqueous layers re-extracted successively with three lots of 4 ml. ethyl acetate, centrifuging each time for 10 min. and adding the ethyl acetate layer to the first extract.

The combined ethyl acetate phase is next extracted successively with four lots of 2 ml. 10% (w/v) $Na_2CO_3$. The $Na_2CO_3$ solution is then extracted once with 2 ml. ethyl acetate to remove traces of free 2:4-dinitrophenylhydrazine. It is then neutralized by the dropwise addition of cold (0–4°) conc. HCl until there is no further evolution of $CO_2$.

The neutralized solution is then extracted successively with four lots of 2 ml. ethyl acetate, and the combined ethyl acetate extracts are evaporated to dryness *in vacuo* at temperatures not exceeding 40°.

*Chromatographic procedure.* The dry residue obtained above is dissolved in 0·1–0·3 ml. 0·1 N-NaOH. Phosphate buffer (0·1 M; pH 7·2) is then added until the red colour of the solution disappears (usually 0·5–1·0 ml. is required). In some experiments 0·2 ml. ethanol was added first, but this was found unnecessary. The total volume of NaOH and phosphate buffer added is noted.

0·2 ml. of the solution (or 0·1 ml. if the expected keto acid content is high) is then placed on a strip of Whatman no. 3MM filter paper with a micrometer syringe. The paper is hung in a sealed glass cabinet and the solvent mixture allowed to ascend for about 16 hr. A number of different solvents were tried, and the following mixture finally adopted: *n*-butanol 70 parts, ethanol 10 parts, 0·5 N-$NH_4OH$ 20 parts.

In the case of the synthetic hydrazones 10 $\mu$g., dissolved in 0·02 ml. dilute phosphate buffer, were applied to the paper strips; as acetone 2:4-dinitrophenylhydrazone is insoluble in this medium ethyl acetate was used instead.

*Elution and quantitative estimation of separated hydrazones.* The coloured area on the paper occupied by the given hydrazone is cut out with scissors, and after cutting into small pieces is placed in a sintered-glass micro funnel.

1 ml. 10% $Na_2CO_3$ is added to the funnel, the contents stirred with a glass rod until the paper has disintegrated and then filtered under pressure into a small test tube. The residue on the funnel is re-extracted with a further 1 ml. $Na_2CO_3$.

To the combined filtrates 1 ml. 2 N-NaOH is added, the red colour measured after 15 min. in a 1 cm. cell using an Ilford Spectrum violet 601 filter and compared with a standard curve prepared from known amounts of the corresponding keto acid.

*Colorimetric estimation of keto acids.* Keto acids were also estimated in the blood samples by the method of Friedemann & Haugen (1943), using ethyl acetate as solvent.

## RESULTS

### *Choice of chromatographic solvent*

The final solvents chosen by the Italian workers were butanol-ethanol mixtures (Cavallini *et al.* 1949*a*, *b*; Rossi & Di Leva, 1951). Starting from their work, we have studied a series of mixtures of *n*-butanol, *iso*butanol, *n*-propanol, *iso*propanol and *n*-amyl alcohol with varying amounts of ethanol, water and 0·5 N-$NH_4OH$, and have compared the relative $R_F$ values of our synthetic hydrazones. As mentioned in the Experimental Section, the final solvent which gave the best separation of these hydrazones was a mixture of *n*-butanol, ethanol and 0·5 N-$NH_4OH$. The relative positions taken up by the different pure hydrazones when run in this solvent mixture are shown in Fig. 1 (chromatograms $P_3$ and $P_9$). In these chromatograms the hydrazones were not carried through the full extraction procedure used when assaying blood, but were applied directly to the paper. It will be seen that under these conditions satisfactory resolution of these pure hydrazones is obtained; as is to be expected, the $R_F$ values show slight variation from day to day, due probably to changes in environmental temperature and slight changes in the composition of the solvent mixture. The ranges of

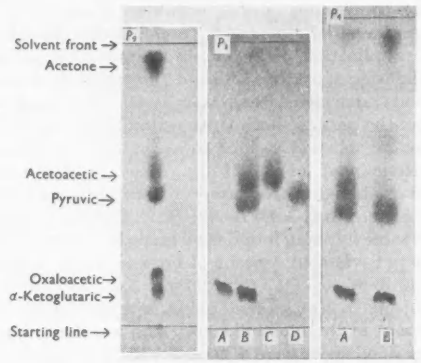


Fig. 1. Chromatograms of pure keto acid 2:4-dinitrophenylhydrazones. $P_9$. α-Ketoglutaric, oxaloacetic, pyruvic and acetoacetic acid hydrazones and acetone hydrazone (10 $\mu$g. each). $P_3$. *A*, 30 $\mu$g. α-ketoglutaric acid hydrazone; *B*, α-ketoglutaric, pyruvic and acetoacetic acid hydrazones (30 $\mu$g. each); *C*, 30 $\mu$g. acetoacetic acid hydrazone; *D*, 30 $\mu$g. pyruvic acid hydrazone. $P_4$. *A*, hydrazones of α-ketoglutaric, pyruvic and acetoacetic acids (30 $\mu$g. each) applied direct to paper; *B*, hydrazones of same acids carried through complete extraction process used for blood analysis.

$R_F$ values found for the different hydrazones are 0·08–0·15 for α-ketoglutaric acid, 0·19–0·29 for oxaloacetic acid, 0·35–0·47 for pyruvic acid, 0·46–0·64 for acetoacetic acid and 0·90–0·95 for acetone.

### *Chromatographic behaviour of synthetic 2:4-dinitrophenylhydrazones*

As subsequent work (see below) showed that oxaloacetic acid is not present in normal human blood in amounts detectable by this technique, the hydrazone of this keto acid was not further studied.

Chromatograms were next prepared by taking the hydrazones through the complete extraction procedure as outlined above for blood. Chromatogram $P_4$ in Fig. 1 shows the results obtained (*A*) when hydrazones were applied directly to the paper, and (*B*) when they were taken through the extraction process used for blood.

It will be seen that the $R_F$ values for the hydrazones of α-ketoglutaric acid and pyruvic acid are unaffected by this treatment, whereas the picture given by acetoacetic acid is profoundly changed;

the spot having an $R_F$ value slightly greater than that of the pyruvic acid hydrazone is largely replaced by a spot just below the solvent front, together with a faint, diffuse spot slightly in front of the original one. It will be noticed, however, that a faint spot also appears just below the solvent front in chromatogram $P_4$ (spot *A*) of Fig. 1, i.e. when the hydrazones are applied directly to the paper. It seemed likely, therefore, that acetoacetic acid hydrazone was undergoing some slight degree of decomposition during the chromatographic procedure, which was markedly accentuated when it was also subjected to the extraction process. Decarboxylation to acetone seemed a likely explanation of these findings. Such decarboxylation might take place to a small extent during the application of the hydrazones to the paper, since a current of hot air from an electric hair drier is directed on to the paper while the hydrazone is being applied in order to prevent undue spreading of the hydrazone solution. When, however, the hydrazone is carried through the full extraction procedure it remains at room temperature in approx. 4% metaphosphoric acid for a short period, after which it is subjected to evaporation *in vacuo* at a temperature of up to 40°. It might be expected, therefore, that further decarboxylation would take place during these procedures. In order to test this possibility, chromatograms were prepared in which the behaviour of the acetoacetic acid hydrazone, applied direct to the paper and also after being carried through the extraction procedure, was compared with that of the acetone hydrazone (Fig. 2). It will be seen that the extracted acetoacetic acid hydrazone has been almost completely converted to a substance giving the same $R_F$ value as the acetone hydrazone.

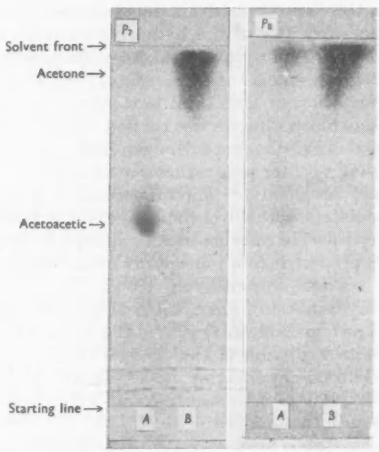


Fig. 2. Chromatographic behaviour of acetoacetic acid 2:4-dinitrophenylhydrazone. $P_7$. *A*, acetoacetic acid hydrazone (30 $\mu$g.) applied direct to paper; *B*, acetone hydrazone (100 $\mu$g.) applied direct to paper. $P_8$. *A*, acetoacetic acid hydrazone (30 $\mu$g.) carried through complete extraction process used for blood analysis; *B*, acetone hydrazone applied direct to paper.

### *Chromatographic behaviour of 2:4-dinitrophenylhydrazones extracted from blood*

Fig. 3 shows two chromatograms, each comparing the picture obtained with blood with that given by the synthetic hydrazones. In paper $P_{10}$ in this figure it will be seen that there are two spots corresponding to the hydrazones of α-ketoglutaric acid and pyruvic acid respectively. A third is visible in the blood picture slightly above the pyruvate spot; the former corresponds in position with that given by the acetoacetate tracer. In addition, it will be noticed that a certain amount of decarboxylation of this hydrazone has taken place, rather more marked in the case of the tracers than with blood, since a fourth spot is visible slightly behind the solvent front. The other chromatogram ($P_2$) in Fig. 3 again shows the α-ketoglutarate and pyruvate hydrazones clearly, but in this case the acetoacetate hydrazone, both in the tracer and in the blood, shows two somewhat indistinct spots just above the pyruvate, in addition to the acetone spot below the

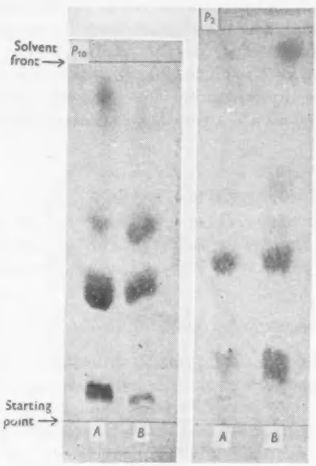


Fig. 3. 2:4-Dinitrophenylhydrazones extracted from human blood. $P_{10}$. *A*, hydrazones of α-ketoglutaric, pyruvic and acetoacetic acids (30 $\mu$g. each) applied direct to paper; *B*, hydrazones extracted from blood. $P_2$. *A*, hydrazones extracted from blood; *B*, 'tracers' (as in $P_{10}$) carried through complete extraction process used for blood analysis.

solvent front. This variable behaviour of the acetoacetate hydrazone will be discussed later.

We have prepared chromatograms from forty blood samples from healthy subjects, and have in each case found (*a*) the spot having the same $R_F$ value as the hydrazone of the α-ketoglutarate, (*b*) the spot with the pyruvate $R_F$ value, and (*c*) a spot or spots which we have provisionally identified as due to acetoacetate and acetone.

### *Identification of the separated blood 2:4-dinitrophenylhydrazones*

Since slight variations in the $R_F$ values of substances separated on paper chromatograms are customarily found, we felt it desirable to obtain further evidence of the identity of these blood spots with the three keto acids mentioned above. An attempt was made, therefore, to compare the absorption curves (in the visible region of the spectrum) of the eluted blood spots with those of the pure hydrazones. The measurements were carried out in a Unicam D.G. spectrophotometer.

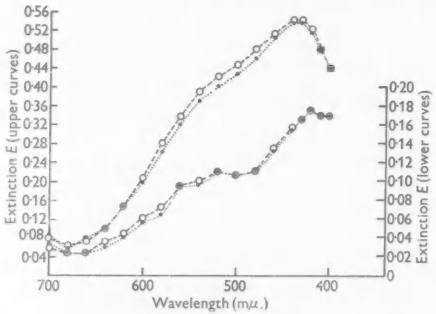


Fig. 4. Absorption curves of 2:4-dinitrophenylhydrazones extracted from 'pyruvic' and 'α-ketoglutaric' spots, compared with curves obtained with pure pyruvic and α-ketoglutaric acid hydrazones. Upper curves, pyruvic acid; lower curves, α-ketoglutaric acid. ○- - -○, pure hydrazone; ●. . .●, extracted material.

The curves obtained with pyruvic and α-ketoglutaric acid hydrazones are shown in Fig. 4, from which it will be seen that the agreement between the extinctions obtained with the eluted and with the pure hydrazones is reasonably good. The curves for the two hydrazones agree well with those obtained by Friedemann & Haugen (1943). These results may be taken to support our conclusion, based on the $R_F$ values, that these spots in the blood chromatograms are due to α-ketoglutaric and pyruvic acid hydrazones respectively.

On the other hand, we were unable to obtain good agreement between the curve of pure acetoacetic acid hydrazone and that of the solution eluted from the blood spot presumed, from evidence described above, to be due to this hydrazone. This lack of agreement may be due to the blood spot containing traces of the acetone hydrazone formed by decarboxylation of the acetoacetic acid derivative while running on the paper.

### *Quantitative estimation of chromatographically separated blood keto acids*

The coloured spots identified as corresponding to the pyruvic and α-ketoglutaric acid derivatives were next extracted with 10% sodium carbonate. After addition of sodium hydroxide to the resulting solutions they were estimated colorimetrically by comparison with the appropriate standard curve prepared from the pure hydrazone of the acid.

As is to be expected there are differences in the colour intensities per unit weight of the hydrazones of the three keto acids in alkaline solution; thus, the colour intensities of the hydrazones formed from equal weights of pyruvic acid, acetoacetic acid and α-ketoglutaric acid are in the ratio of 1·00:0·74:0·48.

Estimation of the 'total keto acids' in the respective blood samples was carried out both by the method of Friedemann & Haugen (1943) and also by applying to filter paper a measured amount of the extract prepared for chromatography, eluting it immediately without chromatographic separation, and estimating colorimetrically against a standard curve prepared from pure pyruvic acid hydrazone. The results obtained on blood samples from fifteen normal subjects in the post-absorptive state are shown in Table 1. Owing to the varying degrees of decarboxylation of the acetoacetic acid hydrazone in different experiments no attempt was made to extract and estimate the amount of this acid present in the samples.

From Table 1 it will be seen that the level of the total keto acids as estimated in the final concentrate applied to the papers for chromatography (column *B* of Table 1) is rather less than the level as estimated by the method of Friedemann & Haugen (1943), amounting to 63–105% (mean 84%) of the latter. This discrepancy is probably due to slight losses in the lengthy extraction and elution process necessary for obtaining the final concentrate for chromatography.

The level of pyruvic acid in the different samples varied from 0·26 to 0·69 mg./100 ml., with a mean of 0·44 mg./100 ml. Pyruvic acid accounts, therefore, for from 42 to 75% (mean 60%) of the total keto acids present in the final concentrate. The level of α-ketoglutaric acid varied from 0·13 to 0·24 mg./100 ml. with a mean of 0·16 mg./100 ml.

Assuming that acetoacetic acid is the only other acidic hydrazone-forming substance present in normal blood (and no other acidic hydrazones are visible by this technique) the approximate level of this acid in blood may be obtained by difference.

Table 1. *Blood keto acid levels in normal subjects in the post-absorptive state*

(Results expressed as mg. keto acid/100 ml. blood; total keto acids expressed as pyruvic acid.)

| Subject | Total keto acids: *A.* Method of Friedemann & Haugen (1943) | Total keto acids: *B.* As estimated in final concentrate applied to paper for chromatography | Pyruvic acid | α-Ketoglutaric acid |
|---|---|---|---|---|
| 1 | 0·79 | 0·57 | 0·34 | — |
| 2 | 1·23 | 1·07 | 0·63 | 0·24 |
| 3 | 1·02 | 0·82 | 0·62 | — |
| 4 | 0·69 | 0·62 | 0·26 | — |
| 5 | 0·77 | 0·74 | 0·37 | 0·13 |
| 6 | 0·79 | 0·58 | 0·33 | — |
| 7 | 0·65 | 0·46 | 0·24 | 0·13 |
| 8 | 0·59 | 0·37 | 0·26 | — |
| 9 | 0·95 | 0·89 | 0·52 | 0·16 |
| 10 | 1·14 | 0·89 | 0·52 | — |
| 11 | 1·05 | 0·89 | 0·55 | — |
| 12 | 0·73 | 0·54 | 0·39 | 0·16 |
| 13 | 0·99 | 1·05 | 0·69 | — |
| 14 | 0·88 | 0·74 | 0·44 | — |
| 15 | 0·73 | 0·66 | 0·44 | — |
| Mean | **0·87** | **0·73** (=84% of *A*) | **0·44** | **0·16** |
| S.E. | ±0·19 | ±0·21 | ±0·14 | |

Thus, in Table 1 the mean level of total keto acids, expressed as pyruvic acid, is 0·73 mg./100 ml., as determined colorimetrically in the concentrate prepared for chromatography and estimated against a standard curve prepared from pure pyruvic acid hydrazone. Of this amount, pyruvic acid accounts for 0·44 mg./100 ml. There is also present 0·16 mg. α-ketoglutaric acid/100 ml., estimated against a standard curve prepared from the pure hydrazone of this acid; when estimated against pure pyruvic acid hydrazone the α-ketoglutaric acid level is equivalent in colour intensity to 0·08 mg. pyruvic acid/100 ml. Therefore, the mean acetoacetic acid concentration = 0·73 − (0·44 + 0·08) = 0·21 mg. pyruvic acid/100 ml.

As pointed out above, however, the hydrazone formed from 1 mg. pyruvic acid yields the same colour intensity as does the hydrazone formed from 1/0·74 mg. acetoacetic acid, so that the concentration of the latter acid in blood = 0·21 × 1/0·74 = 0·28 mg. acetoacetic acid/100 ml. blood.

Table 2. *Mean concentrations of keto acids in post-absorptive human blood*

| | As mg. of each keto acid/100 ml. blood | As mg. pyruvic acid/100 ml. blood |
|---|---|---|
| Pyruvic acid | 0·44 | 0·44 |
| α-Ketoglutaric acid | 0·16 | 0·08 |
| Acetoacetic acid | 0·28 | 0·21 |
| | Total keto acids | **0·73** |

The mean concentrations of these three keto acids in post-absorptive human blood are therefore as shown in Table 2. Pyruvic acid is therefore the most plentiful of the three keto acids which we have demonstrated in human blood under post-absorptive conditions.

*Effect of exercise on blood pyruvate level*

Johnson & Edwards (1937) and Lu (1939*b*) have demonstrated that in exercise there is a considerable elevation of the blood pyruvate level, estimated by Peters & Thompson's (1934) modification of the method of Case (1932). By isolating the pyruvic acid 2:4-dinitrophenylhydrazone from 400 ml. of blood drawn from four subjects after they had run to exhaustion, Johnson & Edwards (1937) concluded that most of the keto acid accumulation in the blood was in fact due to pyruvic acid or to some unstable precursor.

It seemed of interest, therefore, to estimate the chromatographically separated keto acids from blood samples taken from four healthy subjects before and after exercise (running up and down stairs till exhausted). The results obtained (Table 3 and Fig. 5) confirmed Johnson & Edwards's earlier conclusion that the excess keto acid present after exercise is very largely pyruvic acid; in our experiments pyruvic acid accounted for 83–99% of the total keto acids accumulating. The excess pyruvic acid is clearly shown in the chromatogram (Fig. 5); it will also be seen from this figure that there is some increase in acetoacetic acid, whereas the α-ketoglutaric acid is not noticeably changed in amount.

Table 3. *Increase in pyruvic acid and total keto acids in blood after exercise*

(Results expressed as mg. pyruvic acid/100 ml.)

| Subject | Increase in total keto acids: Method of Friedemann & Haugen (1943) | Increase in total keto acids: As estimated in final concentrate applied to the paper for chromatography | Increase in pyruvic acid | Pyruvic acid as % of total keto acids |
|---|---|---|---|---|
| 1 | 0·55 | 0·40 | 0·36 | 90 |
| 2 | 1·81 | 1·84 | 1·53 | 83 |
| 3 | 1·25 | 1·14 | 1·13 | 99 |
| 4 | 1·63 | 1·47 | 1·45 | 99 |

## DISCUSSION

The results described above confirm the earlier findings of the Italian workers that it is possible to separate and identify both pyruvic and α-ketoglutaric acids in extracts of human blood by means of paper chromatography. Our identification of these acids rests primarily on the agreement between the $R_F$ values of pure 2:4-dinitrophenylhydrazones of these keto acids and the values obtained on the same chromatogram for the two most slowly running spots in the blood extracts. Rough measurements of the absorption curves given by the extracted hydrazones in alkaline solution support this identification.

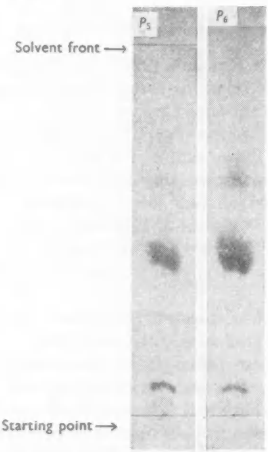


Fig. 5. Effect of exercise on blood keto acids. $P_5$. 2:4-Dinitrophenylhydrazones extracted from 0·7 ml. blood *before* exercise. $P_6$. Hydrazones extracted from 0·5 ml. blood *after* exercise.

Rossi & Di Leva (1951) observed two further spots in the blood extracts, which they did not identify. We have regularly found one or more spots moving more rapidly than the pyruvic acid hydrazone in post-absorptive human blood. We think that the most slowly moving of these spots is acetoacetic acid hydrazone, but owing to decarboxylation of this compound another rapidly moving spot of acetone hydrazone also sometimes appears just behind the solvent front. The intensity of this acetone spot differs noticeably in different chromatograms. We feel that varying degrees of decarboxylation of the acetoacetic acid hydrazone also take place while the hydrazone is actually travelling up the paper; thus, whereas the spots due to α-ketoglutaric and pyruvic acids are in most cases small and show little evidence of spreading, the acetoacetate spot is in nearly all cases markedly elongated, with an indefinite and gradually fading upper edge.

As mentioned earlier, we have not obtained any evidence of the presence of oxaloacetic acid in blood in amounts detectable by this technique. In this connexion it is of interest to note that Frohman, Orten & Smith (1951) reported that the level of oxaloacetic acid in rat blood is less than 0·1 mg./100 ml., while LePage (1950) concluded that if oxaloacetic acid is present in rat blood it is below the level of detection by his method. Our failure to detect oxaloacetic acid in human blood is, in our opinion, not due to its decarboxylation to pyruvic acid during the extraction. Thus, when a solution of pure oxaloacetic acid hydrazone is placed on the paper, a single spot with a low $R_F$ value is found; when it is carried through the full extraction procedure some decarboxylation evidently occurs, since a spot with an $R_F$ value the same as that of the pyruvate hydrazone is also present; this, however, is always less intense than the unchanged oxaloacetate spot; if it is present in blood, therefore, one would expect to find an oxaloacetate spot, which under our conditions is not present.

The mean level of pyruvic acid in blood which we have estimated colorimetrically after chromatographic separation from the other keto acids, is considerably greater than that reported by the Italian workers, and amounts to about 60% of the

total keto acids. As our figures for the acetoacetic acid content of post-absorptive blood are obtained by difference, they will include the variable amount of acetone formed by decarboxylation during the chromatographic run. Our mean value of 0·28 mg. acetoacetic acid/100 ml. blood agrees well with the range of values (0·08–0·40 mg./100 ml.) found in five healthy subjects by Stark & Somogyi (1943), although it is somewhat higher than the five normal values (0·06–0·26 mg./100 ml.) reported by Rosenthal (1949). Our mean estimated level for α-ketoglutaric acid in blood also agrees reasonably well with the value of 0·21 mg./100 ml. found by Cavallini *et al.* (1949*b*).

Although the estimation of acetoacetic acid by this procedure is inaccurate when applied to blood owing to its instability and low concentration, it is possible, we feel, to obtain a more exact estimate of the pyruvate level by means of preliminary chromatographic separation than by the Friedemann & Haugen technique. In studying the changes in the level of blood keto acids in disease it not only provides us with a more specific method for the estimation of pyruvic acid, but also affords a means of detecting visually and of estimating roughly keto acids other than pyruvic acid, particularly if these are present in raised amounts as a result of the disease.

## SUMMARY

1. A method is described for the separation and identification of blood keto acids by paper chromatography, and for the extraction and quantitative estimation of the separated pyruvic and α-ketoglutaric acids.

2. In post-absorptive blood from healthy human subjects pyruvic acid, α-ketoglutaric acid and acetoacetic acid are the only keto acids detectable by this method. Under these conditions pyruvic acid accounts for about 60 % of the total keto acids.

3. The rise in the level of blood keto acids due to exercise has been shown to be almost entirely due to pyruvic acid, although the acetoacetate level is also increased.

We wish to thank Dr F. H. Brain for his valuable advice and help in the synthesis of some of the keto acids studied in the course of this work. Our thanks are also due to our colleagues who provided blood samples, and to Dr C. J. Earl and Mr D. H. Burchett for their help in some of the blood pyruvate estimations.

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# The Molecular Structure and Shape of Yeast Glycogen

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Since the recognition of glycogen in yeast by Errera (1885) there have been a number of attempts to isolate it and to compare its nature with that of various animal glycogens. The earlier work used optical rotation, opalescence of aqueous solution, colour with iodine and differences in mineral, phosphorus and nitrogen contents of the samples, as a basis for comparison. Jeanloz (1944) pointed out that comparisons of this nature were unsuitable for detecting possible differences among glycogens and he extended the studies by fractionation of the glycogen by electrodialysis, viscosity determinations of the triacetate, and measurement of the percentage hydrolysis brought about by wheat $\beta$-amylase.

In the work to be described an attempt has been made to obtain more precise information concerning the structure of yeast glycogen. Methylation and periodate oxidation were used to determine the type of linkage of the glucose radicals and the average chain length. The position of the branching point in the unit chain was found by a study of the extent of hydrolysis with pure crystalline $\beta$-amylase. A comparison of this amylolysis limit with that produced by a non-crystalline $\beta$-amylase, containing a trace of Z enzyme, was used to indicate the probable absence of $\beta$-1:3 links in the chains. In addition, some idea of the shape of the glycogen molecule has been obtained by viscosity measurements. A comparison of samples of the glycogen prepared by different methods of extraction has also made it possible to offer an explanation of the findings of various workers which seemed to indicate the presence of at least two chemically and functionally different glycogens in the yeast cell (Ling, Nanji & Panton, 1925; McAnally & Smedley-Maclean, 1937; Trevelyan & Harrison, 1952).

## EXPERIMENTAL AND RESULTS

### *Methods and materials*

The yeast, general analyses for N and P, and the paper-chromatographic methods used for the sugars were the same as those described previously (Northcote & Horne, 1952). The paper chromatograms of the methylated glucose derivatives were run for 12 hr. at room temperature. The rabbit-liver glycogen was kindly given by Dr D. J. Bell; it had a chain length of 12 by periodate oxidation (Bell & Manners, 1951).

### *Isolation of the glycogen*

Three methods were adopted based on preparations previously described in the literature. In each case 36 g. of the same sample of pressed baker's yeast were used.

(A) *Cytolysis with* 3% NaOH. The method of Bell & Northcote (1950) was used followed by extraction of the glycogen from the insoluble residue and the alkaline solution.

(i) Extraction from the insoluble residue. This was heated at 75° with 700 ml. 0·5 N-acetic acid for 2 hr.; pH of the mixture approx. 2. The mixture was centrifuged and the solid washed with water at 75° and recentrifuged. The washings were repeated until the supernatant gave no precipitate with ethanol or colour with $I_2$. The extract and washings were combined and precipitated with ethanol. The glycogen obtained was purified, until its N content was less than 0·1%, by solution in water and precipitation with ethanol.

(ii) Extraction from the alkaline solution. The solution was made just acid with acetic acid and any insoluble material removed by centrifugation. The supernatant was

Table 1. *Glycogen prepared by three methods from baker's yeast*

(The ash, N and P contents of all the samples of glycogen were less than 0·1%. The glycogen was dried at 100° over $P_2O_5$ at 0·01 mm. Hg for 6 hr. in each case.)

| | Glycogen extracted from insoluble residue (i) | | Glycogen in solution (ii) | |
|---|---|---|---|---|
| | Yield (% dry wt.) | $[\alpha]_D^{18°}$ in water | Yield (% dry wt.) | $[\alpha]_D^{18°}$ in water |
| (A) NaOH-cytolysed pressed yeast | 3·2 | +184° (*c*, 0·76; *l*, 2 dm.) | 0·0 | — |
| (B) NaOH-cytolysed dried yeast | 2·0 | +184° (*c*, 0·5; *l*, 2 dm.) | 0·8 | +186° (*c*, 0·8; *l*, 2 dm.) |
| (C) Mechanically broken yeast | 0·0 | — | 1·5 | +188° (*c*, 1·1; *l*, 2 dm.) |

evaporated under reduced pressure at 60° to 20 ml. and the polysaccharides precipitated with ethanol. The subsequent purification of the glycogen followed the method of Daoud & Ling (1931).

(B) *Use of dried yeast.* The drying was carried out at 15° for 48 hr. in a vacuum desiccator (0·01 mm. Hg) over $P_2O_5$. The procedures were then those described for A (i) and A (ii).

(C) *Cytolysis by grinding with fine sand.* The method of Harden & Young (1912) was used. The grinding was thorough and maintained for 45 min. and the mass poured directly into boiling water (500 ml.) and extracted for 2 hr. The solution was decanted and the residue re-extracted with 250 ml. of boiling water. The solution and the washings were combined and the glycogen subsequently isolated from the solution and the insoluble residue by the procedures described for A (i) and A (ii).

The yields and characteristics of the material obtained by these methods are given in Table 1.

### *Acid hydrolysis of the glycogen*

Each sample of glycogen (100 mg.) was hydrolysed for 3 hr. on a boiling-water bath with 10 ml. N-HCl. The hydrolysates were neutralized with $Ag_2O$ and the excess Ag removed by $H_2S$ in the usual manner. The resultant solutions were clarified by charcoal, concentrated under reduced pressure to 5 ml. and investigated separately on paper chromatograms. Glucose was the only sugar found. The glucose content of each sample of glycogen was estimated by the anthrone method as used by Seifter, Dayton, Novic & Muntwyler (1950); it was never less than 98·5%.

### *Methylation of the glycogen*

The glycogen (A (i), 5 g.) was first acetylated then simultaneously deacetylated and methylated according to the method of Haworth & Percival (1932). Eight successive methylations were performed and the solid material was finally extracted with boiling $CHCl_3$, dried over anhyd. $MgSO_4$, concentrated under reduced pressure and the methylated polysaccharide precipitated by light petroleum, b.p. 40–60°.

The yield of acetylated glycogen was 7·4 g. (83% of theory). $[\alpha]_D^{17°}$ +158° in $CHCl_3$ (*c*, 1·0; *l*, 2 dm.). (Found: $CH_3CO$, 44·5%.) The yield of methylated glycogen was 4·4 g. (83·5% from 7·4 g. of the acetate). $[\alpha]_D^{17°}$ +200° in $CHCl_3$ (*c*, 1·0; *l*, 2). (Found: OMe, 45·0%.)

### *Hydrolysis of the methylated glycogen*

The material (2·0 g.) was refluxed with 50 ml. of methanol containing 2% (w/w) HCl for 12 hr. After removal of the HCl by treatment with $Ag_2CO_3$ and removal of the solid by filtration, the resulting solution was evaporated under reduced pressure. The residual syrup, dissolved in N-HCl, was heated under reflux on a boiling-water bath for 12 hr. The solution was freed from HCl by an excess of $Ag_2CO_3$ in the customary manner and concentrated under reduced pressure to a syrup in the presence of $BaCO_3$ (Bell, 1944).

### *Identification and separation of the cleavage products*

The solution was subjected to a chromatographic separation by the method of Bell (1944) using a 3 g. column of silica gel prepared according to Tristram (1946). Three fractions were obtained, two of which, the tetra- and trimethyl fractions, crystallized. Yield: $Me_4$, 0·180 g.; $Me_3$, 1·356 g.; $Me_2$, 0·417 g.; aggregate yield 89·5%. The average chain length calculated on the molecular ratios of methylated sugars recovered is 11·7 glucose units/non-reducing end group.

The tetramethyl fraction had m.p. 93°, not depressed on admixture of an authentic specimen of 2:3:4:6-tetramethyl glucose (m.p. 95°). $[\alpha]_D^{20°}$ +83°, equilibrium value in water (*c*, 1·8; *l*, 2 dm.). (Found: OMe, 51·5. Calc. for $C_{10}H_{20}O_6$: OMe, 52·5%.) On the paper chromatogram the whole fraction showed a single compact spot identical with a control sample of 2:3:4:6-tetramethyl glucose.

The whole trimethyl fraction had $[\alpha]_D^{18°}$ +90° in water, falling to +68° (*c*, 2·0; *l*, 2 dm.). The crystals melted at 108° and on admixture did not depress the melting point of authentic 2:3:6-trimethyl glucose (m.p. 113°).

The sample was recrystallized from ether. (Found: OMe 41·1. Calc. for $C_9H_{18}O_6$: OMe, 41·9%.) The rotation of the methylated sugar was depressed to a negative value by solution in methanol containing 2% (w/w) HCl. $[\alpha]_D^{18°}$ +68° initially; $[\alpha]_D^{18°}$ −28° after 24 hr. (*c*, 0·6; *l*, 2 dm.). The whole fraction without recrystallization showed a single compact spot on a paper chromatogram identical with authentic 2:3:6-trimethyl glucose.

The dimethyl fraction showed the presence of at least two components (i.e. two distinct spots) when it was investigated on a paper chromatogram. The probable identity of these was only inferred by comparing them on a paper chromatogram with 2:3- and 3:6-dimethyl glucoses when a correspondence was obtained; the $R_G$ values were 0·55 and 0·48, respectively. It is possible that the presence of 3:6-dimethyl glucose in the hydrolysate results from incomplete methylation of the glycogen.

### *End-group assay of the glycogen by periodate oxidation*

This was carried out by the method of Halsall, Hirst & Jones (1947). Glycogen (A (i), 91·2 mg.) was dissolved in 70 ml. of a solution containing 7 g. KCl and 20 ml. 8% (w/v) sodium metaperiodate solution. A control without glycogen was investigated simultaneously. The solutions were kept agitated on rollers. Samples (10 ml.) were taken at intervals, excess periodate decomposed by the addition of 0·5 ml. ethylene glycol (pH 5 to methyl red), and the formic acid present titrated against 0·01 N-NaOH in $CO_2$-free air with methyl red as indicator. The following results were obtained:

| Time of sampling (hr.) | 48 | 168 | 192 |
|---|---|---|---|
| Titre (ml. 0·0104 N-NaOH) | 0·46 | 0·64 | 0·64 |

The titre, 0·64 ml. of the alkali, is equivalent to 2·14 mg. of formic acid and corresponds to a unit chain length of 12 glucose radicals. The experiment was repeated using glycogen *c*, 378·7 mg. of which in 120 ml. of solution yielded 9·77 mg. of formic acid, corresponding to a chain length of 11·2 glucose units.

### *Hydrolysis of the glycogen by β-amylase*

The enzyme was prepared by extracting barley flour (100 g.) with water (300 ml.) for 1 hr. The resulting solution after dialysis contained α- and β-amylases and maltase. α-Amylase activity was destroyed by storage at pH 3·6 and

4° for 1 week; β-amylase was isolated by ethanolic fractionation of the solution according to the method of Halsall, Hirst, Hough & Jones (1949).

The freeze-dried enzyme contained no maltase and failed to hydrolyse starch beyond 60% (possible maltose) on prolonged incubation, thus indicating the absence of α-amylase. Small amounts of Z enzyme (Peat, Pirt & Whelan, 1952*a*) were present, as shown by the hydrolysing action of this preparation on yeast glucan and on laminarin (Manners, 1952*a*).

Crystalline β-amylase (sweet potato) (Balls, Thompson & Walden, 1946; Balls, Walden & Thompson, 1948) was kindly given by Dr C. S. Hanes, F.R.S. This enzyme was free from Z-enzyme activity.

The action of β-amylase on rabbit-liver glycogen and yeast glycogen (A (i) and B (ii)) was studied. About 30 mg. of the polysaccharide, dissolved in 2 ml. of water, were treated with 5 ml. of 0·2 M-sodium acetate buffer (pH 4·6), and 3 ml. of enzyme solution (approx. 0·1 mg./ml.). This mixture was incubated at 25° under toluene and 2 ml. samples were withdrawn at intervals for determination of the reducing power by the method of Shaffer & Somogyi (1933) as modified by Hanes & Cattle (1938). The results obtained with β-amylase-containing Z enzyme are shown in Fig. 1. In all cases the enzyme was active at the end of the experiment, as shown by an increase in reducing power on the addition of a small amount of starch solution. Additional digests were set up using yeast glycogen with the following modifications:

(*a*) Previous incubation of the glycogen with emulsin (commercial B.D.H. preparation) (Peat, Thomas & Whelan, 1952).

(*b*) The non-crystalline β-amylase was preheated at 60° at pH 4·8 for 30 min. to destroy Z enzyme (Peat, Pirt & Whelan, 1952*b*).

(*c*) The buffer used was changed to pH 3·6 to inhibit the action of Z enzyme (Peat *et al.* 1952*a*).

In (*b*) and (*c*) three separate additions of enzyme after 96 hr. intervals were needed to reach the hydrolysis limit. The results for the above experiments are summarized in Table 2.

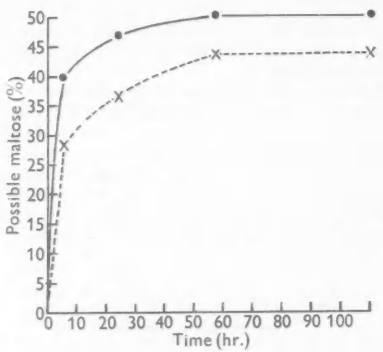


Fig. 1. Hydrolysis of yeast and rabbit-liver glycogens by barley β-amylase, pH 4·6; temp. 25°. ●—●, yeast glycogen A (i); ×- - -×, rabbit-liver glycogen.

### *The viscosities of yeast glycogen and rabbit-liver glycogen*

The determinations were made at 21° in a horizontal capillary viscosimeter (Tsuda, 1928; Ostwald, 1933). The capillary had the following characteristics: radius, 0·0222 cm.; length, 28·60 cm.; flow volume, 1·128 ml. The yeast glycogen used was preparation A (i) and the concentrations of the aqueous solutions varied over the range 0·4–2·0%. Fig. 2 shows the results obtained at constant pressure (47·9 cm. of water). The viscosity of the yeast glycogen was also measured at varying pressures and it was found to be independent of the rate of shear (range investigated 290–2100 sec.$^{-1}$). Thus the flow of this solution is strictly Newtonian.

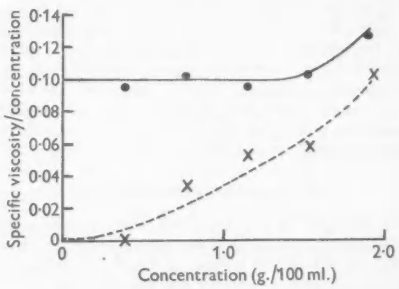


Fig. 2. Viscosities of yeast and rabbit-liver glycogens in aqueous solution at 21°. ●—●, yeast glycogen; ×- - -×, rabbit-liver glycogen.

Table 2. *Extent of hydrolysis of glycogen by β-amylase at 25°*

| | Percentage possible maltose at hydrolysis limit | | |
|---|---|---|---|
| | Yeast glycogen | | |
| | A (i) | B (ii) | Rabbit-liver glycogen |
| Crystalline β-amylase, pH 4·6 | 49 | 50 | 43 |
| Crystalline β-amylase + emulsin, pH 4·6 | 50 | 50 | — |
| Barley β-amylase + Z enzyme, pH 4·6 | 50 | 50 | 43 |
| Barley β-amylase + Z enzyme, pH 3·6 | 49 | — | — |
| Barley β-amylase (after heating to 60°, pH 4·8) | 50 | — | — |

*The sorption of water vapour by rabbit-liver glycogen and yeast glycogen A (i)*

The glycogens were dried at 100° over $P_2O_5$ at 0·01 mm. Hg to constant weight. Approximately 0·2 g. portions were allowed to come to equilibrium at 26° with water vapour in closed desiccators containing $H_2SO_4$ solutions of various known concentrations (Wilson, 1921). Equilibrium was reached with the low relative water-vapour-pressure systems in about 5 days; with high humidities about 9 days were necessary. The amount of water taken up by the glycogens was obtained by direct weighing, using well stoppered weighing bottles. In all cases the amount of water taken up by the two glycogens was very similar (Table 3). A detailed study of the moisture sorption isotherms of these glycogens will be reported separately.

Table 3. *The sorption of water vapour by yeast and rabbit-liver glycogens at 26°*

| Relative humidity (%) | Water sorbed (g./100 g. dry wt. of glycogen) | |
|---|---|---|
| | Yeast glycogen | Rabbit-liver glycogen |
| 5·0 | 2·9 | 3·1 |
| 38·5 | 9·7 | 9·9 |
| 68·0 | 14·9 | 15·2 |
| 91·0 | 32·2 | 31·2 |

## DISCUSSION

The methods used for the extraction of yeast glycogen have been of two main types: (*a*) mechanical breakage of the cell by grinding with fine sand (Harden & Young, 1912; Stockhausen & Silbereisen, 1936; Jeanloz, 1944), (*b*) alkaline cytolysis using either dilute (2–3%) sodium hydroxide solution (Salkowski, 1894; Ling *et al.* 1925) or 30–60% sodium hydroxide solution (McAnally & Smedley-Maclean, 1937; Trevelyan & Harrison, 1952). Method (*b*) gives rise to some glycogen in solution and an insoluble residue containing glycogen; the latter can be rendered soluble by pretreatment with cold N-HCl (McAnally & Smedley-Maclean, 1937) or by acetic acid as in the present work (see also Bell & Northcote, 1950). Use of 3% sodium hydroxide solution on pressed baker's yeast failed to bring any of the glycogen into solution. Ling *et al.* (1925) used dried brewer's yeast and obtained glycogen in the dilute sodium hydroxide solution. It has been shown in the work described here that when the baker's yeast is dried some glycogen can be rendered soluble by treatment with dilute sodium hydroxide solution. It seems likely from the microscopical studies on the yeast cell wall preparations (Northcote & Horne, 1952) that all of the glycogen is freed from the cell wall by a complete mechanical breakage of the cell. It has been shown that some glycogen is rendered soluble in water by the incomplete breakage which is obtained by grinding yeast with sand. The insolubility of the glycogen in the sodium hydroxide preparations would appear to be due to an envelopment of the glycogen by the insoluble glucan membrane and therefore to be mechanical rather than chemical. Concentrated alkali and strong acids will subsequently affect the permeability of the membrane, allowing the glycogen to diffuse. If the glycogen is polydisperse (Bridgeman, 1942; Bell, Gutfreund, Cecil & Ogston, 1948; Record, 1948) it is not surprising that some of the molecules can pass into solution through the glucan membrane while others are held back. It is known that drying affects the permeability of the yeast membrane (Thorsell & Myrbäck, 1951) and thus a ready explanation is afforded for the solubility of the glycogen after the yeast has been dried.

The chemical analysis of the polysaccharide shows that for the most part it is very similar to rabbit-liver glycogen. Its chain length, as given by end-group assay of the methylated polysaccharide and periodate oxidation, is 11 or 12 glucose units. The relative amounts of tetra-, tri- and di-methylglucoses obtained after hydrolysis of the methylated product shows that the majority of the links within the glycogen are of the 1:4 type. The analysis resembles that obtained by Hirst, Hough & Jones (1949) for rabbit-liver glycogen and the similarity of the chromatograms of the dimethyl fractions probably indicates that the interchain links of the two glycogens are of the same type. The values of the specific rotations obtained for yeast glycogen and the acetylated and methylated products were of the order to be expected for a polysaccharide in which the glucosidic links are α, and this was further supported by the enzymic investigation discussed below. The hydrolysis of the yeast polysaccharide by β-amylase, which is specific for 1:α:4-glucosidic links, gives 50% of the possible maltose. Jeanloz (1944) has already reported a hydrolysis limit, using wheat amylase, of 48% on one of the fractions of yeast glycogen obtained by electrodialysis. These amylolysis limits are about 5% higher than for most glycogens (Manners, 1952*b*) and therefore show a slight difference in the position of the branching points of the chains from those of animal glycogens (Meyer & Fuld, 1941). Z enzyme and emulsin have no action on the glycogen and thus the anomalous links which occur in amyloses are probably absent. In both the chemical and enzymic work, when comparisons were made between the samples of the yeast glycogen prepared by the various methods, no differences could be detected, and thus there appears to be only one type of glycogen in the yeast cell. This glycogen functions as a normal storage

substance and not as a structural unit of the cell wall (Northcote & Horne, 1952).

Because of the apparent similarity between the chemical structure of rabbit-liver glycogen and yeast glycogen it is interesting to note the significant difference in their viscosities (Fig. 2). This difference indicates a more asymmetric nature of the yeast polysaccharide (the intrinsic viscosity of the liver glycogen is almost zero and hence this molecule is approximately spherical in shape), or a difference in the degree of hydration, or a combination of both of these factors. Some idea of the extent of hydration of the two polysaccharides in solution may be obtained from the moisture sorption experiments. These show a surprising degree of similarity and therefore may indicate a similarity in the degree of hydration in solution. The value of the intrinsic viscosity of the yeast glycogen calculated from the values shown in Fig. 2 is 0·102 (S.D. = 0·010). Since the flow is Newtonian an estimate of the axial ratio of the yeast glycogen molecule can be made from Simha's equation (Simha, 1940; Mehl, Oncley & Simha, 1940) taking the partial specific volume of the glycogen to be 0·65 (Bell *et al.* 1948) and correcting for hydration according to Oncley (1941). The ratio obtained is approximately 8:1 if the shape is considered as a prolate ellipsoid of revolution, or 1:13 as an oblate ellipsoid of revolution.

## SUMMARY

1. Samples of yeast glycogen have been prepared by various methods. A comparison of the products has failed to give any evidence for the existence of more than one glycogen in yeast. An explanation is put forward to account for the insolubility of part of the yeast glycogen when the cell is cytolysed by sodium hydroxide solution.

2. Yeast glycogen has been shown to be composed of glucose molecules linked for the most part by 1:α:4 links in unit chains of approximately 12 glucose radicals.

3. The hydrolysis limits obtained by the action of β-amylase have indicated some slight difference in the position of the branching point in yeast glycogen and in rabbit-liver glycogen. No anomalous links hydrolysable by Z enzyme or emulsin have been found in yeast glycogen.

4. The viscosity measurements indicate an asymmetric shape for the yeast glycogen molecule compared with that of rabbit-liver glycogen. An axial ratio indicating this asymmetry has been calculated.

I wish to express my gratitude to Dr D. J. Bell for his interest and advice in connexion with this work and to Dr T.-C. Tsao for assistance with the viscosity determinations.

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# The Amino-acid Sequence in the Glycyl Chain of Insulin

## 1. THE IDENTIFICATION OF LOWER PEPTIDES FROM PARTIAL HYDROLYSATES

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Insulin has been shown to be composed of two types of polypeptide chains held together by —S—S— bridges. These chains may be separated after breaking the —S—S— bridges by oxidation to —$SO_3H$ groups (Sanger, 1949*a*). The more acidic glycyl chain is found in the fraction (*A*) which is soluble at pH 6·5 and the more basic phenylalanyl chain in the insoluble fraction (*B*). By making use of the methods of Consden, Gordon & Martin (1947) for fractionating peptides on paper chromatograms, Sanger & Tuppy (1951*a*, *b*) were able to determine the complete amino-acid sequence of the phenylalanyl chain. This and the following paper describe a similar investigation on fraction *A* to determine the amino-acid sequence in the glycyl chain.

End-group analysis of fraction *A* showed that it contained about twenty amino-acid residues (Sanger, 1949*a*) and this was in reasonable agreement with the value of 2900 for the molecular weight which was obtained in the ultracentrifuge (Gutfreund & Ogston, 1949). The amino-acid composition was considerably simpler than that of insulin or fraction *B* since it contained no arginine, histidine, lysine, phenylalanine, threonine or proline. By studying the dinitrophenyl (DNP) peptides produced on partial hydrolysis of the DNP derivative of fraction *A* it was shown that the *N*-terminal sequence was Gly.Ileu.Val.Glu.Glu (Sanger, 1949*b*).

The abbreviations used and the methods of writing peptide structures are the same as those given by Sanger & Tuppy (1951*a*).

## METHODS

### *Preparation of fraction A*

When the fractions of oxidized insulin were prepared as previously described (Sanger, 1949*a*) the tyrosine residues were to some extent oxidized to a product of unknown structure, which was distinguished from tyrosine by its $R_F$ value in butanol-acetic acid and was referred to as TyrX (Sanger & Tuppy, 1951*a*). This increased the complexity of the chromatograms since, for every tyrosine-containing peptide, two spots were present, one with tyrosine and one with TyrX. In the original method a large excess of performic acid was used to oxidize the cystine residues. It has now been found that if only a slight excess is used the formation of TyrX can be avoided. The yield of the fraction *A* is somewhat lower, but for most purposes this drawback is offset by the advantage of having a simpler peptide mixture.

Insulin (1 g.) was dissolved in 8 ml. formic acid and treated with 0·48 ml. 30% (w/w) $H_2O_2$ (1·6 equiv. on the basis of the S content). After standing for 1 hr. 8 ml. water were added to decompose the performic acid. Subsequent treatment and preparation of the fractions was as described before (Sanger, 1949*a*). The yield of fraction *A* was 0·16–0·22 g. and of fraction *B* 0·20–0·27 g. No TyrX could be detected in either fraction.

### *Hydrolysis and preliminary fractionation*

In the experiments described in this paper, hydrolysis was carried out in 12 N-HCl at 37°, the most generally useful conditions for obtaining a large number of small peptides. Hydrolysates prepared by the action of 0·1 N-HCl or N-NaOH at 100° were also studied, but they are not described as no new peptides were found in them, and they were not investigated in great detail.

To effect preliminary group fractionation of the hydrolysate prior to paper chromatography several methods have been studied in different experiments. An 'aromatic' fraction ($A_{1a}$) could be obtained by adsorption on charcoal. This method did not give a completely clear-cut separation since the fraction contained considerable quantities of the longer non-aromatic peptides besides those of tyrosine. However, it was sufficiently simple to study by paper chromatography and the method was useful for the initial removal of aromatic peptides, which do not fractionate well by ionophoresis in silica gel.

About half the residues in fraction *A* are acidic and there are no basic amino-acids. The majority of the peptides are therefore acidic and it was found that the most useful methods for group separations were those which fractionate the peptides according to the different number of charged groups they contain.

In a preliminary experiment the peptides were fractionated on the ion-exchange resin, Amberlite IR-4 B (Consden, Gordon & Martin, 1948; Sanger & Tuppy, 1951*a*). This separated the hydrolysate into three clear-cut fractions containing peptides of cysteic acid, other acidic peptides and neutral peptides, respectively. In general, however, ionophoretic methods were preferred since they are easier to carry out and separate the hydrolysate into a larger number of simpler fractions. Although better separations are usually obtained by ionophoresis on filter paper, it is not convenient to work with sufficient material on this support-

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ing medium. We have therefore used silica jelly as in the original method of Consden, Gordon & Martin (1946). Difficulties due to adsorption are encountered in this method with peptides containing basic or aromatic amino-acids; the former were absent from fraction *A*, the latter had been removed by adsorption on charcoal.

*Experiment* $A_1$. This is given as a typical experiment in which all the peptides detected are recorded. It is described as a single experiment, although much of the data was in fact obtained using other fractions from experiments in which slightly different methods for preliminary separations

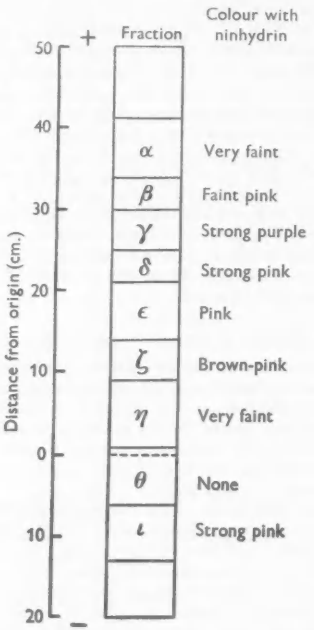


Fig. 1. Ionophoretic fractionation of unadsorbed peptides from partial hydrolysate of fraction *A* in 0·05 M-ammonium acetate (Exp. $A_1$).

had been employed. Fraction *A* (100 mg.) was hydrolysed for 3 days at 37° with 5 ml. 12 N-HCl. After removal of the excess acid by repeated evaporation *in vacuo*, the residue was dissolved in 4 ml. 5% (w/v) acetic acid and stirred for 20 min. with 150 mg. charcoal (British Drug Houses Ltd., previously washed well with 20% (w/v) acetic acid). The charcoal was filtered off and washed with 2 ml. 5% acetic acid. The combined filtrates, containing the unadsorbed peptides, were taken to dryness and fractionated by ionophoresis as described below. The adsorbed peptides were eluted from the charcoal with 50 ml. 5% phenol in 20% acetic acid to give fraction $A_{1a}$. For the ionophoresis of the unadsorbed peptides the method (C 1) of Consden *et al.* (1946) was used. The jelly, which was strengthened with filter-paper pulp, was prepared by neutralizing 140 ml. 8% sodium silicate solution with 5% acetic acid (115 ml.). After setting it was washed overnight with 0·05 M-ammonium acetate to replace the $Na^+$ ions by $NH_4^+$ ions. The material to be fractionated was applied in a gutter near the cathode. The jelly was covered by a glass lid weighted to prevent uneven shrinking and puddling of the electrolyte. A potential of 220 V. was applied for 19 hr. At the end of this period a 'print' of the jelly was taken on to a sheet of filter paper, which was sprayed first with 1% methanolic KOH to remove $NH_3$, and then with 5% methanolic acetic acid followed by 0·1% ninhydrin in butanol. As the resolution was satisfactory, the jelly was cut out according to the marker sheet and the cuts dried *in vacuo* over $H_2SO_4$ and NaOH. The amino-acids and peptides were eluted from the dried jelly with 5% acetic acid. The solution was then filtered and evaporated to dryness. Fig. 1 is a diagram of the ionophoresis and shows the bands obtained. Each fraction was then investigated by paper chromatography.

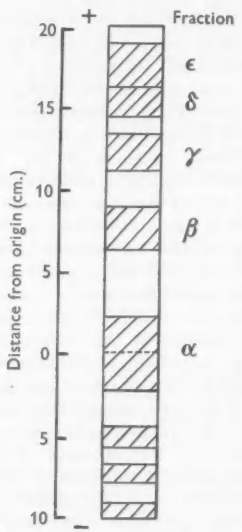


Fig. 2. Ionophoretic fractionation of cysteic acid peptides from partial hydrolysate of fraction *A* in 0·2 N-acetic acid (Exp. $A_2$).

*Experiment* $A_2$. Several peptides containing more than one cysteic acid residue were present in the hydrolysate of fraction *A*, and these could not easily be fractionated on paper chromatograms as they moved so slowly in all solvents studied. An attempt was therefore made to separate them by ionophoresis. Since the $-SO_3H$ group is more acidic (pK = approx. 1·3; Andrews & Schmidt, 1927) than the —COOH group (pK = 2·5) it is possible, by choosing an electrolyte of suitable pH, to find conditions where the latter are virtually uncharged whereas the former are ionized. Consden *et al.* (1946) made use of this property to separate cysteic acid from aspartic and glutamic acids with 0·02 N-HCl as electrolyte. We have found that good separations of cysteic acid peptides can be obtained by ionophoresis in 0·2 N-acetic acid (pH 2·75). Under these conditions all peptides with no $-SO_3H$ group move towards the cathode (due to the fully charged $-NH_3$ group); those with

one $-SO_3H$ group are approximately neutral and a very clear-cut fraction ($A_{2a}$) with only this type of peptide can be obtained. Peptides with more than one cysteic acid residue move towards the anode, and in the case of those from fraction *A* can readily be fractionated. Free cysteic acid, which contains an unusually strong α-COOH group, also moves towards the anode.

Ionophoresis on paper was found to be very effective for this purpose, and the technique of Durrum (1950) was superior to several others that were investigated. This is probably due largely to the flow gradient of electrolyte up the paper, which tends to sharpen the bands. Since acetic acid is not itself an effective buffer it was important to remove free and bound acid from the hydrolysate and to apply the material at a relatively low concentration.

Fraction *A* was hydrolysed for 2 days with 12 N-HCl at 37°, and the excess acid removed by repeated evaporation *in vacuo*. The residue was taken up in water and excess $Ag_2O$ added to eliminate the bound HCl. After centrifuging and washing the precipitate with water, the solution was taken to dryness. It was applied in a concentration of 100 mg./ml. as a narrow band at the apex of the filter paper (Whatman no. 3, 56 × 25 cm.). After wetting the paper with electrolyte a potential of 220 V. was applied for 16 hr. The distribution of the bands, which was revealed by spraying a marker strip with ninhydrin, is shown in Fig. 2. They were cut out and eluted from the paper, taken to dryness and investigated as described in the next section.

For large 'cuts', such as those obtained in this experiment, a more convenient method of elution than that described by Sanger & Tuppy (1951*a*) was to allow the eluates to drop on to a sheet of celluloid or directly on to the polythene strip, thereby eliminating the need for constant watching during the elution.

### *Examination of the peptides*

The various fractions obtained in experiment $A_1$ were investigated by paper chromatography using essentially the same methods reported by Sanger & Tuppy (1951*a*). Usually Whatman no. 3 filter paper has been used since it has a high capacity and gives good resolutions. The solvent system phenol-0·3 % $NH_3$, which was used extensively, will be referred to throughout simply as 'phenol', and *m*-cresol-0·3 % $NH_3$ will be referred to as 'cresol'. With the exception of leucine and isoleucine, all the amino-acids present in fraction *A* (see Table 13) can be separated by chromatography using phenol. One-dimensional chromatograms were therefore used to identify the amino-acids present in the hydrolysates of the various peptides. Butanol-benzyl alcohol-HCN was used to distinguish between leucine and isoleucine.

To determine the *N*-terminal residues of the peptides, the DNP method has been used in most cases. Sanger & Tuppy (1951*a*) carried out the condensation between the peptide and 1:2:4-fluorodinitrobenzene (FDNB) in the presence of $NaHCO_3$. The salt had to be removed before paper chromatography could be carried out and this was achieved by extraction of the DNP peptide into ethyl acetate. In order to avoid this somewhat tedious step the reaction is now carried out in the presence of trimethylamine, which may be removed *in vacuo*. The identity of the *N*-terminal residue was deduced by Sanger & Tuppy from its absence in the hydrolysate of the DNP peptide. This clearly is less decisive than the identification of the actual DNP amino-acid present, and interpretation is especially difficult where tyrosine is present. In most cases we have therefore identified the DNP derivatives using buffered paper chromatograms (Blackburn & Lowther, 1951; Biserte & Osteaux, 1951; Monnier & Penasse, 1950). Sufficient material for such chromatograms could usually be obtained and, since the amino-acids present in the peptides were already known, it was generally possible to obtain an unequivocal result.

The peptide eluted from two chromatograms on Whatman no. 3 filter paper was dissolved in 0·1 ml. 1 % trimethylamine and to this was added a solution of 0·01 ml. FDNB in 0·2 ml. ethanol. After standing for 2 hr., a few drops of water and trimethylamine solution were added and the excess FDNB extracted three times with ether. The residue, after evaporation of the aqueous solution to dryness, was taken up in three drops 5·7 N-HCl into a capillary tube. After 8 hr. heating at 105°, the yellow hydrolysate was diluted with twice its volume of water and the DNP amino-acid extracted into ether (three times). Both the ether extract and the aqueous solution were evaporated to dryness *in vacuo*. In general, the residue from the aqueous solution was chromatographed in phenol. DNP-cysteic acid and *O*-DNP-tyrosine are the only water-soluble DNP derivatives encountered with fraction *A*. DNP-cysteic acid has an $R_F$ value of 0·52. *O*-DNP-tyrosine travels at the solvent front and because of the dirty material collected there and its weak grey colour with ninhydrin, it is not easy to detect. Using butanol-acetic acid, both DNP-cysteic acid ($R_F = 0{\cdot}46$) and *O*-DNP-tyrosine ($R_F = 0{\cdot}84$) can be detected, but overlapping of the amino-acids, aspartic acid, serine, glycine, and possibly glutamic acid, then occurs.

The DNP amino-acids can mostly be resolved using *tert.*-amyl alcohol as solvent (Blackburn & Lowther, 1951). The paper (Whatman no. 1) and solvent were both treated with a phthalate buffer at pH 6. The chromatograms were preferably developed in the dark at a constant temperature (20°) (Biserte & Osteaux, 1951). The $R_F$ values obtained with this system were not constant and control spots of DNP amino-acids were always run simultaneously. Unless otherwise stated, the DNP amino-acids recorded in Tables 1–10 were identified using this solvent system.

In some of the earlier experiments, before the description of the above solvent system was published, use was made of the same solvent but a different buffer mixture (phosphate, pH 6·75) which had been developed by Partridge & Davis (unpublished). This did not give such good results as the phthalate buffer. Another solvent mixture suggested by Partridge & Davis was prepared from equal volumes of water, benzene and acetic acid, and could be used with unbuffered paper. It was useful for the DNP amino-acids with low $R_F$ values, such as DNP-cysteic acid, DNP-aspartic acid, DNP-glutamic acid and DNP-serine.

A disadvantage with paper chromatograms of DNP derivatives are the yellow artifacts which are also formed. The main ones are probably 2:4-dinitroaniline and 2:4-dinitrophenol. The dinitroaniline travels near the front and does not complicate the interpretation of results. The dinitrophenol spot is usually very strong and is accompanied by two other spots which travel at very similar rates to some of the DNP amino-acids. These artifact spots can be distinguished and removed, however, by exposure of the paper to HCl vapour, when they lose their yellow colour.

The identification of bis-DNP-tyrosine and some of the faster-moving DNP derivatives was somewhat unsatis-

Table 1. *Peptides from fraction* $A_{1a}$

| Spot no. (Figs. 3 and 4) | Amino-acids present | Strength of amino-acid after Hydrolysis | Strength of amino-acid after DNP treatment | DNP amino-acid identified | Structure* |
|---|---|---|---|---|---|
| 1 | $CySO_3H$ | × × | | | |
| | Glu | × | | | [$CySO_3H$, Glu, Ala] |
| | Ala | × | | | |
| 2 | $CySO_3H$ | × × × | | | |
| | Asp | × × × | | | [$CySO_3H$, Asp, Glu, Tyr] |
| | Glu | × × × | | | |
| | Tyr | × × | | | |
| 3 | $CySO_3H$ | × | | | |
| | Asp | × | | | [$CySO_3H$, Asp, Glu, Tyr] |
| | Glu | × | | | |
| | Tyr | × | | | |
| 4 | $CySO_3H$ | × × | × × | | |
| | Asp | × × | ? | Asp† | Asp.[$CySO_3H$, Tyr] |
| | Tyr | × × | ×‡ | | |
| 5 | $CySO_3H$ | × × × | × × | | |
| | Asp | × × × | × | Asp | Asp.[$CySO_3H$, Asp, Tyr] |
| | Tyr | × × × | ×‡ | | |
| 6 | $CySO_3H$ | × × | × | | |
| | Asp | × × | × | Asp | Asp.[$CySO_3H$, Asp, Tyr] |
| | Tyr | × × | ×‡ | | |
| 7 | $CySO_3H$ | × × | × × | | |
| | Tyr | × × | ?‡ | Tyr | Tyr.$CySO_3H$ |
| 8 | $CySO_3H$ | × × | | | |
| | Glu | × × | | | |
| | Gly | × | | Gly | Gly.[$CySO_3H$, Glu, Val, Leu] |
| | Val | × × | | | |
| | Leu | × × | | | |
| 9 | Asp | × | ? | | |
| | Tyr | × | ×‡ | Asp | Asp.Tyr |
| 10 | Asp | × × | ? | | |
| | Tyr | × × | ×‡ | Asp | Asp.Tyr |
| 11 | Asp | × × | × × | | |
| | Glu | × × | × | Glu | Glu.[Asp, Tyr] |
| | Tyr | × × | ×‡ | | |
| 12 | Glu | × × | × × | | |
| | Tyr | × × | ?‡ | Tyr | Tyr.Glu |
| 13 | Tyr | × × × | | | Tyrosine |
| 14 | Asp | × × | | | |
| | Tyr | × × | | | [Asp, Tyr] |
| 15 | Glu | × × × | | | |
| | Gly | × | | | |
| | Val | × × | | | [Glu, Gly, Val, Leu] |
| | Leu | × × | | | |
| 16 | Glu | × × × | | | |
| | Val | × × | | | [Glu, Val, Leu] |
| | Leu | × × | | | |
| 17 | Glu | × × | | | |
| | Tyr | × × | | | [Glu, Tyr, Leu] |
| | Leu | × × | | | |
| 18 | Glu | × | | | |
| | Gly | × | | | |
| | Val | × | | | Gly.[Glu, Val, Leu] ($A_{1/3}$) |
| | Leu | × | | | |

Table 1 (*cont.*)

| Spot no. (Figs. 3 and 4) | Amino-acids present | Strength of amino-acid after Hydrolysis | Strength of amino-acid after DNP treatment | DNP amino-acid identified | Structure* |
|---|---|---|---|---|---|
| 19 | Ser | × × × | × | Ser† | Ser.[Tyr, Leu] |
| | Tyr | × × × | ×‡ | | |
| | Leu | × × × | × × | | |
| 20 | Glu | × × | × | Leu | Leu.[Glu, Tyr] |
| | Tyr | × × | ×‡ | | |
| | Leu | × × | — | | |
| 21 | Tyr | × × | | | [Tyr, Leu] |
| | Leu | × × | | | |
| 22 | Glu | × | × | Ser | Ser.[Glu, Tyr, Leu] |
| | Ser | × | ? | | |
| | Tyr | × | § | | |
| | Leu | × | × | | |
| 23 | Glu | × × | | | Leu.[Glu, Val] ($A_{17d}$) |
| | Val | × × | | | |
| | Leu | × × | | | |

* Square brackets indicate that the sequence of amino-acids inside the brackets has not been established.
† Identified using benzene-acetic acid-water.
‡ Identified as *O*-DNP-tyrosine.
§ *O*-DNP-tyrosine not identified on this phenol chromatogram.

Spots 8, 15, 18 and 19. Yellow colour with ninhydrin.

Spots 7 and 8 overlapped considerably, but as spot 8 gave an initial yellow colour with ninhydrin the separated areas of the spots could be detected and cut out. Better separated using collidine (see Fig. 13). After DNP treatment spot 7 gave a little DNP-glycine in addition to bis-DNP-tyrosine. Spots 11 and 12 often occurred as one spot and resolution could sometimes be achieved by attaching another sheet of filter paper to the bottom of the chromatogram and allowing the butanol-acetic acid to run for a longer time.

Spot 19. Yellow colour with ninhydrin. Sometimes difficult to locate as it was contaminated with red material washed down behind butanol-acetic acid front.

Spot 23 usually contained some tyrosine indicating contamination with some other peptide.

factory on the above solvent systems, since streaking often occurs near the front of the solvent and weak spots are difficult to detect. Better results were obtained with a reversed phase chromatogram using silicone-treated paper (Kritchevsky & Tiselius, 1951). A suitable solvent system was found to be $CHCl_3$ (10 vol.), *n*-propanol (10 vol.) and 1·5 N-acetic acid (6 vol.). After applying the spots to the paper, it was suspended in the tank with the organic phase (lower) and equilibrated for 3 hr. The paper was then dipped in the aqueous phase and the chromatogram allowed to develop. Bis-DNP-tyrosine and bis-DNP-lysine did not move, DNP-leucine ($R_F = 0{\cdot}11$), DNP-valine ($R_F = 0{\cdot}25$) and DNP-phenylalanine ($R_F = 0{\cdot}13$) moved slowly while all other DNP amino-acids and the 'artifact' bands ran faster. The $R_F$ values changed from experiment to experiment especially with the time of equilibration. The DNP amino-acids were therefore always identified by comparison with marker spots rather than by $R_F$ values.

In earlier experiments many of the peptides were subjected to deamination by the method of Consden *et al.* (1947), but these are not recorded since clearer results have since been obtained with the DNP method. Tyrosine was never detected after deamination, but if present in a peptide in a position other than *N*-terminal, two new spots were present, which moved on a phenol chromatogram slightly more slowly than serine and slightly faster than alanine, respectively. These spots, which are possibly nitroso derivatives of tyrosine, were pale yellow before treatment with ninhydrin and gave a greyish colour after treatment. If tyrosine was present as the *N*-terminal residue, these spots were absent or relatively weak and this fact could be used as a fairly reliable guide to the structure of aromatic peptides.

## RESULTS

### *Experiment* $A_1$

The results obtained with the various fractions of experiment $A_1$ are given in Figs. 3–12 and Tables 1–10. Figs. 3–12 are diagrams of the two-dimensional chromatograms. Tables 1–10 give the results obtained with the eluted peptides. In all cases column 2 lists the amino-acids given on complete hydrolysis and the approximate strength is indicated in column 3. (The × 's have the same significance as in the previous paper (Sanger & Tuppy, 1951*a*).) No distinction is made in these tables between leucine and isoleucine which are both referred to as Leu. Column 4 gives the strength of the amino-acid remaining in the aqueous solution after extraction of the hydrolysed DNP peptide solution with ether, and column 5 the DNP amino-acid identified in the ether extract or, in the case of DNP-cysteic acid, in the aqueous solution. The last column gives the structures of the peptides as far as they can be deduced from the data given in the tables.

Two chromatograms of fraction $A_{1a}$ are shown. In one (Fig. 3) the initial solvent was phenol, and in the other (Fig. 4) cresol. With the latter solvent better resolution of some of the faster-moving peptides was obtained. No attempt was made to identify the slower-moving spots.

Besides the peptides given in Table 1, fraction $A_{1a}$ contained appreciable amounts of Ser.Val.$CySO_3H$ ($A_{1\zeta_2}$), Leu.Glu.Leu ($A_{1\delta_6}$) and the free amino-acids cysteic acid, glycine and alanine. These were not present in earlier experiments where less charcoal (100 mg. charcoal/100 mg. fraction $A$ hydrolysate) was used to adsorb the aromatic peptides.

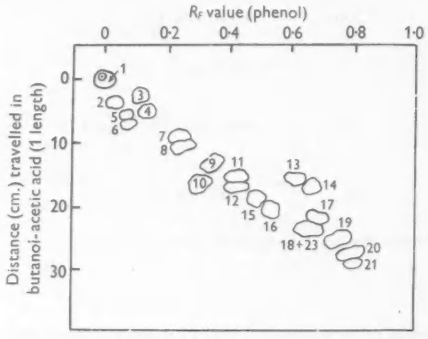


Fig. 3. Chromatogram of fraction $A_{1a}$ using phenol (see Table 1). In Figs. 3–14 the fraction was applied in the position marked by the small dotted circle, and run first in the direction represented horizontally, which was the longer dimension of the filter paper.

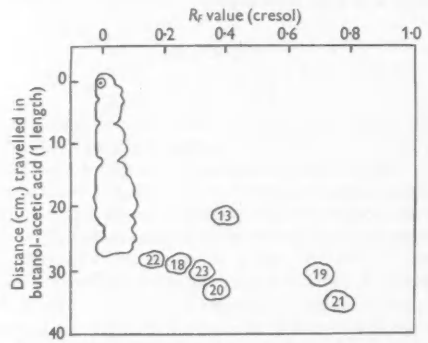


Fig. 4. Chromatogram of fraction $A_{1a}$ using cresol (see Table 1).

The duplication of many of the spots containing aspartic acid and tyrosine may be due to the presence of small amounts of TyrX, different amide contents, or due to different contents of aspartic acid residues. At least three spots (9, 10 and 14) were obtained which gave on hydrolysis aspartic acid and tyrosine and analysed as Asp.Tyr. This suggested that the aspartic acid residue concerned was derived initially from an asparagine residue and spot 14 may in fact be Asp(-$NH_2$).Tyr, as it gives a yellow colour with ninhydrin similar to asparagine. Again, spots 4–6 seem identical in amino-acid composition. The DNP treatment suggests that spots 5 and 6 differ from spot 4, in having a greater proportion of aspartic acid. Spots 1–6 were sometimes eluted together from a phenol chromatogram, which had been developed only one length in each direction, and the material re-run on a second chromatogram using phenol (2 lengths) and butanol-acetic acid ($1\frac{1}{2}$ or 2 lengths). Although much better resolutions were obtained in this way, there was never sufficient material available for a satisfactory end-group determination.

The chromatograms obtained with the ionophoretic fractions ($A_{1a}$–$A_{1\iota}$) are much simpler than that obtained with the aromatic fraction ($A_{1a}$), only a few peptides being present in each. The results with fraction $A_{1\theta}$ are not recorded since it only contained traces of the monoamino-acids and Gly.[Glu, Val, Leu] ($A_{1\eta_3}$).

*Partial hydrolysis of peptide* $A_{1a19}$. Peptide $A_{1a19}$ was further characterized by hydrolysing a sample with 12N-HCl for 3 days at 37°. The hydrolysate was analysed on a strip of Whatman no. 4 filter paper which was developed in only one direction with butanol-acetic acid (1 length). In addition to unchanged peptide and free amino-acids, a yellow spot ($R_F = 0{\cdot}65$) was obtained which yielded only serine and leucine on hydrolysis. It gave a yellow colour with ninhydrin, indicating that the serine was *N*-terminal and that it was therefore Ser.Leu (peptide $A_{1\iota7}$). This established the structure of the original tripeptide ($A_{1a19}$) as Ser.Leu.Tyr. A spot very close to the unchanged tripeptide gave mainly leucine and tyrosine on hydrolysis but also contained some serine. It was probably Leu.Tyr mixed with a little Ser.Leu.Tyr.

*Partial hydrolysis of peptide* $A_{1\delta6}$. This spot had a much lower $R_F$ value in phenol than Glu.Leu ($A_{1\varepsilon8}$) or Leu.Glu ($A_{1\varepsilon7}$). The strength of the amino-acids after DNP treatment suggested two glutamic residues were present. Samples of this peptide were collected and hydrolysed 3 days with 12N-HCl at 37°. The hydrolysate was chromatographed using phenol (1 length) and butanol-acetic acid (1 length) as solvents. Samples of Leu.Glu and Glu.Leu were run as markers during the development with butanol-acetic acid. The results are shown in Table 6. Spot 2 was unchanged tripeptide while spots 3 and 4 corresponded to Leu.Glu and Glu.Leu respectively. This establishes the structure of $A_{1\delta6}$ as Glu.Leu.Glu since it has glutamic acid as the *N*-terminal residue and gives Glu.Leu and Leu.Glu on partial hydrolysis.

### *Experiment* $A_2$

Fraction $A_{2\alpha}$ (Fig. 2), which contained only peptides with one cysteic acid residue, was fractionated by chromatography and the results are shown in Fig. 13 and Table 11.

Samples of fraction $A_{2\beta}$ were chromatographed using phenol (2 lengths) followed by butanol-acetic acid (2 lengths) or collidine (1 length). In both cases

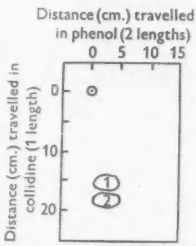


Fig. 5.

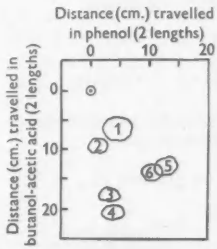


Fig. 6.

Fig. 5. Chromatogram of fraction $A_{1\alpha}$ (see Table 2). When the $R_F$ was low a second folded sheet was clamped to the lower edge of the original sheet and the distance of the spots from the original is then given instead of $R_F$.

Fig. 6. Chromatogram of fraction $A_{1\beta}$ (see Table 3).

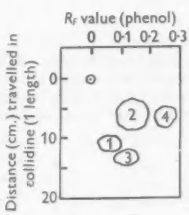


Fig. 7.

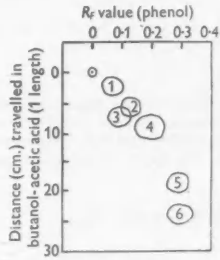


Fig. 8.

Fig. 7. Chromatogram of fraction $A_{1\gamma}$ (see Table 4).

Fig. 8. Chromatogram of fraction $A_{1\delta}$ (see Table 5).

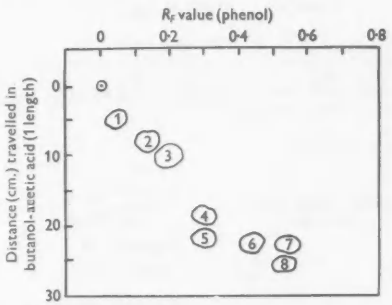


Fig. 9. Chromatogram of fraction $A_{1\epsilon}$ (see Table 7).

one main spot ($A_{2\beta 1}$) was obtained with smaller amounts of three other spots. The main spot gave a yellow colour with ninhydrin and had moved 9 cm. on phenol and 19 cm. on collidine or 12 cm. on phenol and 7·5 cm. on butanol-acetic acid. It contained $CySO_3H$ (× ×), Glu (× ×), Gly (×), Ala (× ×), Val (×), Leu (×).

Fraction $A_{2\gamma}$ was again submitted to ionophoresis using the same conditions to remove some contamination from fraction $A_{2\beta}$. This gave a pure

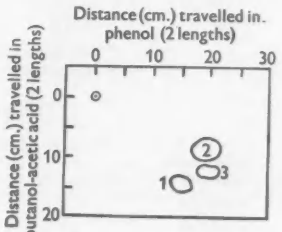


Fig. 10. Chromatogram of fraction $A_{1\zeta}$ (see Table 8).

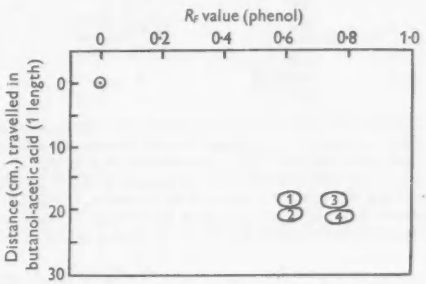


Fig. 11. Chromatogram of fraction $A_{1\eta}$ (see Table 9).

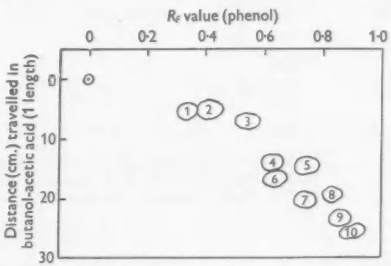


Fig. 12. Chromatogram of fraction $A_{1\iota}$ (see Table 10).

peptide ($A_{2\gamma 1}$) which contained $CySO_3H$ (× × ×), Glu (× ×), Ala (× ×). Glutamic acid was shown to be the *N*-terminal residue, indicating the structure Glu.[$CySO_3H$, $CySO_3H$, Ala].

A sample of this peptide was hydrolysed 3 days with 12 *N*-HCl at 37°, and the hydrolysate chromatographed on a sheet of Whatman no. 4 paper. The results are shown in Fig. 14 and Table 12. Glu.$CySO_3H$ was obtained so that the structure of $A_{2\gamma 1}$ is Glu.$CySO_3H$.[$CySO_3H$, Ala].

Table 2. *Peptides from fraction $A_{1\alpha}$*

| Spot no. (Fig. 5) | Amino-acids present | Strength of amino-acid after: Hydrolysis | Strength of amino-acid after: DNP treatment | DNP amino-acid identified | Structure |
|---|---|---|---|---|---|
| 1 | $CySO_3H$ | × × × | × × × | Glu* | Glu.$CySO_3H$ |
| | Glu | × × × | × | | |
| 2 | $CySO_3H$ | × × × | × | $CySO_3H$† | $CySO_3H$.Asp |
| | Asp | × × × | × × × | | |

* Identified using benzene-water-acetic acid as solvent.
† Identified in aqueous layer using phenol.

Table 3. *Peptides from fraction $A_{1\beta}$*

| Spot no. (Fig. 6) | Amino-acids present | Strength of amino-acid after: Hydrolysis | Strength of amino-acid after: DNP treatment | DNP amino-acid identified | Structure |
|---|---|---|---|---|---|
| 1 | $CySO_3H$ | × × × × | | | Cysteic acid |
| 2 | $CySO_3H$ | × | | | Glu.$CySO_3H$ ($A_{1\alpha 1}$) |
| | Glu | × | | | |
| 3 | Glu | × × × | × | Glu* | Glu.Asp |
| | Asp | × × × | × × × | | |
| 4 | Glu | × × × | | | See footnote |
| 5 | Asp | × × | | | Aspartic acid |
| 6 | $CySO_3H$ | × | | | $CySO_3H$.Ala ($A_{1\gamma 3}$) |
| | Ala | × | | | |

* Identified using benzene-water-acetic acid as solvent.
Spot 1. Spread over extensive area, due to salt or overloading.
Spot 4. Contained only glutamic acid but moves at a very different rate from free glutamic acid or glutamine. It is presumably Glu.Glu known to be present in the *N*-terminal sequence. Its rate, relative to cysteic acid, was similar to that found for the synthetic material by Consden, Gordon & Martin (1949), and the ionophoretic rate was faster than that of glutamic acid, in agreement with the results of these workers. On paper ionophoresis using 0·05 M-ammonium acetate, glutamic acid travelled 6·1 cm. while spot 4 travelled 7·7 cm.

Table 4. *Peptides from fraction $A_{1\gamma}$*

| Spot no. (Fig. 7) | Amino-acids present | Strength of amino-acid after: Hydrolysis | Strength of amino-acid after: DNP treatment | DNP amino-acid identified | Structure |
|---|---|---|---|---|---|
| 1 | $CySO_3H$ | × × | | | Cysteic acid |
| 2 | Asp | × × × | | | Aspartic acid |
| 3 | $CySO_3H$ | × × × | × | $CySO_3H$* | $CySO_3H$.Ala |
| | Ala | × × × | × × × | | |
| 4 | Glu | × × | | | Glutamic acid |

* Identified in aqueous phase using butanol-acetic acid.
Spots 2 and 3 were not well separated when butanol-acetic acid was used as the second solvent.
Spot 3. Yellow colour with ninhydrin turning to grey on longer heating.

Table 5. *Peptides from fraction $A_{1\delta}$*

| Spot no. (Fig. 8) | Amino-acids present | Strength of amino-acid after: Hydrolysis | Strength of amino-acid after: DNP treatment | DNP amino-acid identified | Structure |
|---|---|---|---|---|---|
| 1 | $CySO_3H$ | × × | | | Cysteic acid |
| 2 | Asp | × × | | | Aspartic acid |
| 3 | $CySO_3H$ | × × | | | $CySO_3H$.Ala ($A_{1\gamma 3}$) |
| | Ala | × × | | | |
| 4 | Glu | × × × × | | | Glutamic acid |
| 5 | Asp | × × | × × | | |
| | Glu | × × | × | Leu | Leu.[Asp, Glu] |
| | Leu | × × | — | | |
| 6 | Glu | × × × × | × × | Glu | Glu.[Glu, Leu] |
| | Leu | × × × | × × | | |

Table 6. *Partial hydrolysate of* $A_{1\delta 6}$

| Spot no. | $R_F$ in phenol | Distance moved in butanol-acetic acid (cm.) | Amino-acids present | Strength |
|---|---|---|---|---|
| 1 | 0·18 | 7·3 | Glu | × |
| 2 | 0·26 | 18·2 | Glu | × × |
| | | | Leu | × |
| 3 | 0·53 | 19 | Glu | × × |
| | | | Leu | × × |
| 4 | 0·53 | 21 | Glu | × |
| | | | Leu | × |
| 5 | 0·78 | 20 | Leu | × |

All leucine spots from the chromatogram of these peptide hydrolysates were cut out, washed with acetone to remove excess ninhydrin, eluted and the material rechromatographed using butanol-benzyl alcohol as solvent. Only leucine, and no isoleucine, was present.

Table 7. *Peptides from fraction* $A_{1\epsilon}$

| Spot no. (Fig. 9) | Amino-acids present | Strength of amino-acid after Hydrolysis | Strength of amino-acid after DNP treatment | DNP amino-acid identified | Structure |
|---|---|---|---|---|---|
| 1 | $CySO_3H$ | × | | | Cysteic acid |
| 2 | $CySO_3H$ | × | | | $CySO_3H$.Ala ($A_{1\gamma 3}$) + aspartic acid |
| | Asp | × | | | |
| | Ala | × | | | |
| 3 | $CySO_3H$ | × | | | [$CySO_3H$, Val] ($A_{1\zeta 3}$) + glutamic acid |
| | Glu | × × | | | |
| | Val | × | | | |
| 4 | Asp | × × | | | Leu.[Asp, Glu] ($A_{1\delta 5}$) |
| | Glu | × × × | | | |
| | Leu | × × | | | |
| 5 | Glu | × × × | | | Glu.[Glu, Leu] ($A_{1\delta 6}$) |
| | Leu | × × | | | |
| 6 | Glu | × × × | | | Val.Glu + $A_{1a16}$ (see footnote) |
| | Val | × × | | | |
| | Leu | × | | | |
| 7 | Glu | × × × | × × × | Leu | Leu.Glu |
| | Leu | × × × | × | | |
| 8 | Glu | × × × | × | Glu | Glu.Leu |
| | Leu | × × × | × × × | | |

Spot 6 is probably a mixture of peptide $A_{1a16}$ ([Glu, Val, Leu]) with Val.Glu. In another experiment, in which more charcoal (approx. 1 g./100 mg. hydrolysate) had been used to remove the 'aromatic' fraction, Val.Glu was identified as a single spot in this position on a chromatogram from a similar ionophoretic fraction. Deamination with nitrosyl chloride indicated valine was *N*-terminal.

Table 8. *Peptides from fraction* $A_{1\zeta}$

| Spot no. (Fig. 10) | Amino-acids present | Strength of amino-acid after Hydrolysis | Strength of amino-acid after DNP treatment | DNP amino-acid identified | Structure |
|---|---|---|---|---|---|
| 1 | Glu | × | | | Glutamic acid |
| 2 | $CySO_3H$ | × × × | × × × | Ser | Ser.[$CySO_3H$, Val] |
| | Ser | × × × | × | | |
| | Val | × × × | × × × | | |
| 3 | $CySO_3H$ | × | | | [$CySO_3H$, Val] |
| | Val | × | | | |

Besides the peptides given above, fraction $A_{1\zeta}$ also contained traces of Leu.Glu ($A_{1\epsilon 7}$) and Glu.Leu ($A_{1\epsilon 8}$) which were detected on a chromatogram in which each solvent was run only one length.

Spot 2. Yellow colour with ninhydrin.

Spot 3 gave a very faint colour with ninhydrin, and could more easily be detected by its fluorescence in ultraviolet light. Deamination with nitrosyl chloride indicated that valine was probably the *N*-terminal residue.

Table 9. *Peptides from fraction* $A_{1\eta}$

| Spot no. (Fig. 11) | Amino-acids present | Strength of amino-acid after Hydrolysis | Strength of amino-acid after DNP treatment | Structure |
|---|---|---|---|---|
| 1 | Glu | × | | Leu.Glu ($A_{1\epsilon7}$) |
| | Leu | × | | |
| 2 | Glu | × | | Glu.Leu ($A_{1\epsilon8}$) |
| | Leu | × | | |
| 3 | Glu | × | × | Gly.[Glu, Val, Leu] |
| | Gly | × | — | |
| | Val | × | × | |
| | Leu | × | × | |
| 4 | Glu | × × | × × | Leu.[Glu, Val] |
| | Val | × × | × × | |
| | Leu | × × | ? | |

Spot 3. Yellow colour with ninhydrin.

Table 10. *Peptides from fraction* $A_{1\iota}$

| Spot no. (Fig. 12) | Amino-acids present | Strength of amino-acid after Hydrolysis | Strength of amino-acid after DNP treatment | DNP amino-acid identified | Structure |
|---|---|---|---|---|---|
| 1 | Ser | × × | | | Serine |
| 2 | Gly | × × | | | Glycine |
| 3 | Ala | × × × | | | Alanine |
| 4 | Tyr | × × | | | Tyrosine |
| 5 | Val | × × | | | Valine |
| 6 | Ser | × × | ? | Ser | Ser.Val |
| | Val | × × | × × | | |
| 7 | Ser | × × | × | Ser | Ser.Leu |
| | Leu | × × | × × × | | |
| 8 | Leu | × × | | | Leucine |
| 9 | Ser | × | | | Ser.Leu.Tyr ($A_{1a19}$) |
| | Tyr | × | | | |
| | Leu | × | | | |
| 10 | Leu | × × | ? | Leu | Ileu.Val |
| | Val | × × | × × | | |

Spots 6, 7 and 9. Yellow colour with ninhydrin.
Spot 7 was shown to contain leucine and not isoleucine using butanol-benzyl alcohol.

Table 11. *Peptides from fraction* $A_{2\alpha}$

| Spot. no. (Fig. 13) | Strength | Structure | Identical with |
|---|---|---|---|
| 1 | × × | Glu.$CySO_3H$ | $A_{1\alpha1}$ |
| 2 | × × | $CySO_3H$.Asp | $A_{1\alpha2}$ |
| 3 | × | [$CySO_3H$, Asp, Glu, Tyr] | $A_{1a2}$, $A_{1a3}$ |
| 4 | × × | [$CySO_3H$, Asp, Tyr] | $A_{1a4}$, $A_{1a5}$ |
| 5 | × × × | $CySO_3H$.Ala | $A_{1\gamma3}$ |
| 6 | × × | Tyr.$CySO_3H$ | $A_{1a7}$ |
| 7 | × | Gly.[$CySO_3H$, Glu, Val, Leu] | $A_{1a8}$ |
| 8 | × | [$CySO_3H$, Glu, Val, Leu] | — |
| 9 | × × × × | Ser.[$CySO_3H$, Val] | $A_{1\zeta2}$ |

Spots 7 and 8. The glutamic acid spot was stronger than the other amino-acid spots.
Spots 7 and 9. Yellow colours with ninhydrin.
Spot 8. Blue colour with ninhydrin.

Fraction $A_{2\delta}$ was chromatographed using phenol (2 lengths) and collidine (1 length). Two spots were obtained. One ($A_{2\delta 2}$) was free cysteic acid. It had travelled 6·5 cm. in phenol and 16·5 cm. in collidine. The other ($A_{2\delta 1}$) had travelled 1·4 cm. in phenol and 20·7 cm. in collidine. It contained cysteic acid and alanine, and hydrolysis of the DNP derivative gave DNP-$CySO_3H$ (× ×), $CySO_3H$ (× ×), Ala (× ×). Its structure was therefore $CySO_3H$.[$CySO_3H$, Ala].

Fraction $A_{2\varepsilon}$ gave only free cysteic acid on hydrolysis.

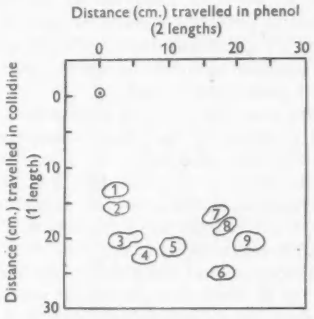


Fig. 13. Chromatogram of fraction $A_{2\alpha}$ (see Table 11).

## DISCUSSION

Since the amino-acid content of fraction *B* is accurately known from the structure determined by Sanger & Tuppy (1951*b*) it is possible to calculate the composition of fraction *A* as the difference between the analytical values for insulin and those for fraction *B*. Using the values collected by Tristram (1949) for the composition of insulin, the figures shown in column 2 of Table 13 are obtained for fraction *A*. In the third column are shown the values obtained in an approximate analysis done by paper chromatography (Sanger, 1949*a*).

### *Peptides of tyrosine*

The various tyrosine peptides are all found in fraction $A_{1a}$. There are four dipeptides containing tyrosine: Tyr.Glu ($A_{1a12}$), Asp.Tyr ($A_{1a9}$, $A_{1a10}$, $A_{1a14}$), Tyr.$CySO_3H$ ($A_{1a7}$) and [Tyr, Leu] ($A_{1a21}$). Since there are only two tyrosine residues in fraction *A* the structure of $A_{1a21}$ must be Leu.Tyr. This is confirmed by the structure of $A_{1a19}$ which was shown by partial hydrolysis to be Ser.Leu.Tyr. Peptide $A_{1a20}$ (Leu.[Glu, Tyr]) cannot be Leu.Glu.-Tyr since Glu.Tyr has not been identified, whereas Tyr.Glu ($A_{1a12}$) has. It must therefore be Leu.Tyr.-Glu and the sequence in fraction *A* becomes Ser.Leu.Tyr.Glu. Peptide $A_{1a22}$ presumably has this structure.

A DNP peptide containing those four amino-acids was detected as a contaminant of a DNP-glycyl peptide in partial hydrolysates of the DNP derivative of fraction *A* (Woolley, 1948; Sanger, 1949*b*). It presumably had the structure Ser.Leu.-(*O*-DNP) Tyr.Glu.

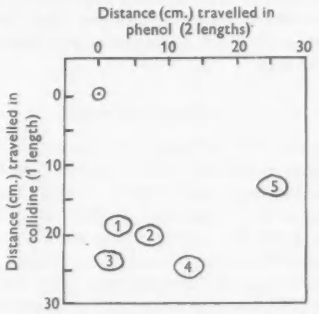


Fig. 14. Chromatogram of partial hydrolysate of peptide $A_{2\gamma 1}$ (see Table 12).

Table 12. *Partial hydrolysis of peptide* $A_{2\gamma 1}$

| Spot. no. (Fig. 14) | Strength | Structure | Identical with |
|---|---|---|---|
| 1 | × × | Glu.$CySO_3H$ | $A_{1a1}$ |
| 2 | × | Cysteic acid | — |
| 3 | × × | $CySO_3H$.[$CySO_3H$, Ala] | $A_{2\delta 1}$ |
| 4 | × × | $CySO_3H$.Ala | $A_{1\gamma 3}$ |
| 5 | × | Glutamic acid | — |

Table 13. *Probable amino-acid composition of fraction A*

| Amino-acid | No. of residues/mol. Calculated from insulin – fraction *B* | No. of residues/mol. Found by approx. analysis |
|---|---|---|
| Cysteic acid | 4 | 4 |
| Aspartic acid | 2 | 2 |
| Glutamic acid | 4* | 4 |
| Serine | 2 | 2 |
| Glycine | 0–1 | 1 |
| Alanine | 1 | 1 |
| Tyrosine | 2 | 2 |
| Valine | 1 | 2 |
| Leucine | 2 | 3 |
| Isoleucine | 1–2 | 1 |

* The figures given by Tristram would indicate a value of 4·5 for the glutamic acid content. Recent analyses (Chibnall & Rees, 1951) make it almost certain that 4 is the correct figure.

Two other dipeptides (Asp.Tyr and Tyr.$CySO_3H$) are not included in the above sequence and must therefore contain the other tyrosine residue. The sequence is therefore Asp.Tyr.$CySO_3H$ and peptide $A_{1a4}$ (Asp.[$CySO_3H$, Tyr]) clearly has this structure. Peptide $A_{1a11}$ has an *N*-terminal glutamic acid residue and also contains aspartic acid and tyrosine.

Its structure is therefore Glu.Asp.Tyr and the sequence in fraction $A$ is Glu.Asp.Tyr.$CySO_3H$. This is confirmed by the presence of Glu.Asp ($A_{1\beta 3}$). The presence of free aspartic acid in the hydrolysate of the DNP derivatives of peptides $A_{1a5}$ and $A_{1a6}$ was taken as evidence that there was a second aspartic acid in these peptides. This would make the above sequence Glu.Asp.Tyr.$CySO_3H$.Asp. However, the evidence is too slight to be certain of this.

### *N-terminal sequence*

Using the DNP technique it was shown that the $N$-terminal sequence was Gly.Ileu.Val.Glu.Glu (Sanger, 1949*b*). Confirmation of this structure is obtained from a number of peptides which are listed in Table 14. Owing to the stability of bonds involving isoleucine and valine many large peptides from this sequence are present, and are mostly found in the fraction adsorbed on charcoal ($A_{1a}$). The two peptides containing one glutamic acid residue (Ileu.Val.Glu and Gly.Ileu.Val.Glu) move rather rapidly on the phenol chromatogram in spot $A_{1a18}$. The two slower-moving spots ($A_{1a16}$, $A_{1a15}$) containing these same amino-acids presumably have two glutamic acid residues.

Since there is only one glycine residue in fraction $A$, any peptide containing glycine must be derived from this $N$-terminal sequence. Such a peptide is $A_{1a8}$. It contains the amino-acids of this sequence and also cysteic acid. Since it moves relatively slowly on a chromatogram, and is found in fraction $A_{2\alpha}$ (spot $A_{2\alpha 7}$) on ionophoresis in acetic acid, the cysteic acid must be an integral part of the peptide, whose structure is therefore Gly.Ileu.Val.Glu.Glu.$CySO_3H$. The presence of Glu.$CySO_3H$ ($A_{1\alpha 1}$) confirms this sequence and peptide $A_{2\alpha 8}$ is presumably Ileu.Val.Glu.Glu.$CySO_3H$. Although the results hitherto discussed do not preclude the presence of another residue of leucine, valine or glutamic acid between the second glutamic acid and the cysteic acid residue in this sequence, it will be seen below that this is in fact not possible.

### *Peptides of alanine*

There is only one alanine residue in fraction $A$ and only one dipeptide was found. This was $CySO_3H$.Ala ($A_{1\gamma 3}$). Alanine was also found in peptides that moved towards the anode in Exp. $A_2$, that is to say in peptides containing two cysteic acid residues. Peptide $A_{2\delta 1}$ was $CySO_3H$.[$CySO_3H$, Ala] and peptide $A_{2\gamma 1}$ Glu.$CySO_3H$.[$CySO_3H$, Ala]. Clearly two sequences are possible Glu.$CySO_3H$.$CySO_3H$.Ala or Glu.$CySO_3H$.Ala.$CySO_3H$. If the second structure were correct Ala.$CySO_3H$ should be detected in the hydrolysate. Consden & Gordon (1950) have shown it to be stable, for it was isolated from a 10-day partial hydrolysate of wool. They also showed that it could be separated from $CySO_3H$.Ala by ionophoresis or chromatography with collidine. In spite of many attempts, no evidence for the presence of this peptide in the fraction $A$ hydrolysate could be obtained and it is therefore concluded that the former sequence (Glu.$CySO_3H$.$CySO_3H$.Ala) is correct. Similarly, Glu.$CySO_3H$.Ala, which would be expected from the second sequence, was never detected. Unfortunately it has not been possible to obtain direct evidence for the presence of $CySO_3H$.$CySO_3H$. It would be expected to move slowly on chromatograms and is probably present in spot $A_{1a1}$ with $CySO_3H$.$CySO_3H$.Ala and Glu.$CySO_3H$.$CySO_3H$.Ala. On ionophoresis in acetic acid it is probably in fraction $A_{2\varepsilon}$ with cysteic acid.

The fact that Glu.$CySO_3H$ is present in the $N$-terminal position of the above sequence (Glu.$CySO_3H$.$CySO_3H$.Ala) and in the $C$-terminal position in the sequence Gly.Ileu.Val.Glu.Glu.$CySO_3H$ suggests that the two are joined together. This is confirmed from the composition of peptide $A_{2\beta 1}$, which contained the amino-acids of the $N$-terminal sequence and also alanine. Since it moved towards the anode on ionophoresis in acetic acid, it must contain the two adjacent cysteic acid residues and its structure is therefore Gly.Ileu.Val.Glu.Glu.$CySO_3H$.$CySO_3H$.Ala.

### *Other peptides*

Three sequences have so far been deduced:

(1) Gly.Ileu.Val.Glu.Glu.$CySO_3H$.$CySO_3H$.Ala.
(2) Ser.Leu.Tyr.Glu.
(3) Glu.Asp.Tyr.$CySO_3H$.

Apart from the valine peptides derived from sequence 1 there are three peptides containing valine: Ser.Val ($A_{1\iota 6}$), Val.$CySO_3H$ ($A_{1\zeta 3}$) and Ser.[$CySO_3H$, Val] ($A_{1\zeta 2}$). Since there are certainly not more than two valine residues in fraction $A$ these must all come from the same sequence: Ser.Val.$CySO_3H$. This structure may also be derived from considering the peptides of serine.

Aspartic acid is present in sequence 3 and also in the peptides $A_{1\delta 5}$ (Leu.[Asp, Glu]) and $A_{1\alpha 2}$ ($CySO_3H$.Asp). Since there are only two aspartic acid residues in fraction $A$ and since the above two peptides cannot come from the same sequence, peptide $A_{1\delta 5}$ must be joined to the $N$-terminal end of sequence 3, which thus becomes Leu.Glu.Asp.Tyr.$CySO_3H$. Leu.Glu is found in peptide $A_{1\varepsilon 7}$.

Peptide $A_{1\delta 6}$ was shown by partial hydrolysis to be Glu.Leu.Glu. Since there are only four glutamic acid residues this must be joined on to sequence 3 to give Glu.Leu.Glu.Asp.Tyr.$CySO_3H$. Any other way of fitting the sequences together would require at least five glutamic acid residues.

Table 14. *Peptides obtained in partial hydrolysate of fraction A of insulin*

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Dipeptides | Ileu.Val ($A_{1\text{-}10}$) | Glu.$CySO_3H$ ($A_{1\text{-}31}$) | Ser.Leu ($A_{1\text{-}7}$) | Glu.Leu ($A_{1\text{-}8}$) | Asp.Tyr ($A_{1\text{-}9}$) | Ser.Val ($A_{1\text{-}0}$) | $CySO_3H$.Asp ($A_{1\text{-}3}$) | |
| | Val.Glu ($A_{1\text{-}4}$) | | Leu.Tyr ($A_{1\text{-}15}$) | Leu.Glu ($A_{1\text{-}17}$) | Tyr.$CySO_3H$ ($A_{1\text{-}19}$) | Val.$CySO_3H$ ($A_{1\text{-}2}$) | | |
| | Glu.Glu ($A_{1\text{-}24}$) | $CySO_3H$.Ala ($A_{1\text{-}26}$) | Tyr.Glu ($A_{1\text{-}13}$) | Glu.Asp ($A_{1\text{-}28}$) | | | | |
| Higher peptides | Ileu.Val.Glu ($A_{1\text{-}5}$) | $CySO_3H$.$CySO_3H$.Ala ($A_{2\text{-}21}$) | Ser.Leu.Tyr ($A_{1\text{-}10}$) | Leu.Glu.Asp ($A_{1\text{-}9}$) | | Ser.Val.$CySO_3H$ ($A_{1\text{-}2}$) | | |
| | Gly.Ileu.Val.Glu ($A_{1\text{-}3}$) | Glu.$CySO_3H$.$CySO_3H$.Ala ($A_{2\text{-}7}$) | Leu.Tyr.Glu ($A_{1\text{-}30}$) | Glu.Asp.Tyr ($A_{1\text{-}11}$) | | | | |
| | Ileu.Val.Glu.Glu ($A_{1\text{-}16}$) | | | | Asp.Tyr.$CySO_3H$ ($A_{1\text{-}8}$) | | | |
| | Gly.Ileu.Val.Glu.Glu ($A_{1\text{-}15}$) | | | Glu.Leu.Glu ($A_{1\text{-}0}$) | | | | |
| | Ileu.Val.Glu.Glu.$CySO_3H$ ($A_{2\text{-}6}$) | | Ser.Leu.Tyr.Glu ($A_{1\text{-}32}$) | | Glu.Asp.Tyr.$CySO_3H$ ($A_{1\text{-}8}$) | | | |
| | Gly.Ileu.Val Glu.Glu.$CySO_3H$ ($A_{1\text{-}8}$, $A_{2\text{-}7}$) | | | | | | | |
| | Gly.Ileu.Val.Glu.Glu.$CySO_3H$.$CySO_3H$.Ala ($A_{2\text{-}21}$) | | | | | | | |
| Sequences in fraction *A* | Gly.Ileu.Val.Glu.Glu.$CySO_3H$.$CySO_3H$.Ala | | Ser.Leu.Tyr.Glu.Leu.Glu.Asp.Tyr.$CySO_3H$ | | | Ser.Val.$CySO_3H$ | $CySO_3H$.Asp | |

In the three sequences deduced there are still five glutamic acid residues so that one must be common to two sequences. This can only be the *C*-terminal residue of sequence 2 and the *N*-terminal residue of sequence 3, which are therefore joined together, and the structure in fraction *A* becomes Ser.Leu.Tyr.Glu.Leu.Glu.Asp.Tyr.$CySO_3H$. Both the 'Leu' residues in this sequence are leucine and not isoleucine since peptides $A_{1\iota7}$ (Ser.Leu) and $A_{1\delta6}$ (Glu.Leu.Glu) contain no isoleucine. The presence of peptide $A_{1a17}$ ([Glu, Tyr, Leu]), which was different from $A_{1a20}$ (Leu.Tyr.Glu), can readily be understood from this sequence. A number of structures are possible, such as Tyr.Glu.Leu or Leu.Tyr.Glu.Leu.Glu.

All the peptides encountered in this work are now accounted for in terms of the four sequences:

(1) Gly.Ileu.Val.Glu.Glu.$CySO_3H$.$CySO_3H$.Ala.
(2) Ser.Leu.Tyr.Glu.Leu.Glu.Asp.Tyr.$CySO_3H$.
(3) Ser.Val.$CySO_3H$.
(4) $CySO_3H$.Asp.

All the amino-acids known to be present in fraction *A* are present in these sequences and there is one too many cysteic acid residues. The $CySO_3H$.Asp must therefore be joined to the *C*-terminal end of sequence 2 or 3, but it is not possible to decide conclusively which.

In conclusion, the various peptides identified are listed in Table 14 with the sequences deduced from them.

## SUMMARY

1. Fraction *A* of oxidized insulin has been subjected to partial hydrolysis with acid and the resulting peptides fractionated by charcoal adsorption, ionophoresis and paper chromatography.

2. It is concluded that the following sequences are present in this fraction:

Gly.Ileu.Val.Glu.Glu.$CySO_3H$.$CySO_3H$.Ala,
Ser.Leu.Tyr.Glu.Leu.Glu.Asp.Tyr.$CySO_3H$,
Ser.Val.$CySO_3H$ and $CySO_3H$.Asp.

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# The Amino-acid Sequence in the Glycyl Chain of Insulin

## 2. THE INVESTIGATION OF PEPTIDES FROM ENZYMIC HYDROLYSATES

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In the previous paper (Sanger & Thompson, 1953*a*) four sequences were shown to be present in fraction *A* of oxidized insulin. It was found, as in the case of fraction *B* (Sanger & Tuppy, 1951*a*), that the complete sequence could not be derived from the small peptides produced on acid hydrolysis, due largely to the great lability of the bonds involving the amino groups of the serine residues. It was therefore necessary to investigate the action of proteolytic enzymes, and the present paper describes the results obtained with peptic and chymotryptic hydrolysates. Trypsin was found to be without action on fraction *A*.

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Although, in fact, it was only necessary to consider the structure of one of the peptides ($A_{p5}$) to work out the sequence of fraction *A*, the hydrolysates were investigated in considerable detail, since confirmation was desired and since the results throw some light on the specificity of the enzymes concerned. Also a number of the peptides from these hydrolysates were required to determine the position of the amide groups (Sanger & Thompson, 1953*b*). The action of carboxypeptidase on fractions *A* and *B*, insulin, and acetyl insulin has also been studied since this was used to confirm the presence of asparagine as the *C*-terminal residue of the glycyl chain.

The peptides with reference numbers starting $A_1$ or $A_2$ refer to those described in the previous paper.

## METHODS

Fraction *A* was prepared free of TyrX as previously described (Sanger & Thompson, 1953*a*). The pepsin used was crystalline pig pepsin (Armour Laboratories). The chymotrypsin was prepared by Mr J. Lightbown of the National Institute for Medical Research, The carboxypeptidase was a commercial preparation obtained from Armour Laboratories. Acetylinsulin was prepared by the method of Hughes (1947).

### *Experiment* $A_p$ (*pepsin*)

In a typical experiment 50 mg. fraction *A* were dissolved in 5 ml. 0·1 N-acetic acid and 2 mg. pepsin added. The mixture was incubated at 37° for 48 hr. After boiling to inactivate the enzyme, the solution was centrifuged to remove denatured pepsin and evaporated to dryness *in vacuo*. In some experiments the digestion was carried out in 0·01 N-HCl, but no difference in the peptides obtained could be detected.

No preliminary fractionation was necessary and samples of the hydrolysate were investigated directly on paper chromatograms. Where Whatman no. 4 filter paper was used an amount equivalent to 5 mg. of the original fraction *A* was applied, and for Whatman no. 3 paper 10–12·5 mg.

The peptides were investigated essentially by the methods described in the previous paper.

### *Experiment* $A_c$ (*chymotrypsin*)

Fraction *A* (50 mg.) was dissolved in 5 ml. water, 2·5 mg. chymotrypsin were added and the pH adjusted to 7·5 with dilute $NH_3$. The mixture was incubated at 37° for 24 hr. It was boiled to inactivate the enzyme and evaporated to dryness *in vacuo*. Before application to a chromatogram, the residue was taken up in a small volume of water, and centrifuged to remove insoluble material.

### *Experiments with carboxypeptidase*

In preliminary experiments it was evident that the carboxypeptidase preparation contained traces of chymotrypsin, which were splitting fraction *A* in the chain. In order to avoid this the enzyme was first incubated with di*iso*propyl fluorophosphonate, which is a specific inhibitor for chymotrypsin (Jansen, Nutting, Jang & Balls, 1949). The action of carboxypeptidase was not affected by this reagent. In order to remove the long-chain material, which frequently interfered with the paper chromatograms, the molecular sieve procedure of Thompson & Partridge (1952) was used.

The di*iso*propyl fluorophosphonate was kept as a 0·1 M solution in dry *iso*propanol. When required for use it was brought to 0·001 M by the addition of water. Of a suspension of carboxypeptidase in water (10–15 mg./ml.) 0·05 ml. was run into 1·45 ml. of this inhibitor solution, and, after adjusting the pH to 7·5, the mixture was incubated at 37° for 30 min. The solution was then added to the substrate (fraction *A*, 10 mg.; fraction *B*, 15 mg.; insulin and acetyl insulin, 25 mg.). Samples (0·3 ml.) were withdrawn at various times with a vacuum pipette. These were heated in a boiling-water bath for 5 min. to inactivate the enzyme. To each were added approximately 50 mg. sulphonated polystyrene resin, which was in bead form (15–30 mesh/in.) and had been regenerated before use by successive washings with 2 N-HCl, water, 2 N-NaOH, water, 2 N-HCl and water (Thompson & Partridge, 1952). The mixture was shaken for 1 hr., the supernatant solution decanted and the beads washed twice with distilled water. The amino-acids were then displaced from the resin with 5 N-$NH_3$ (0·2 ml.) and investigated on paper chromatograms.

## RESULTS

### *Experiment* $A_p$ (*pepsin*)

Two chromatograms of the peptic hydrolysate are shown in Figs. 1 and 2. When each solvent was run only one length of the paper (Fig. 1) spots 3–9 were not sufficiently resolved to give clear results. It was therefore necessary to clamp a second folded sheet of paper on to the bottom edge during each run so that the chromatograms were developed two lengths in each direction (Fig. 2). The combined results are shown in Table 1.

*Partial hydrolysis of peptides* $A_{p5}$, $A_{p14}$, $A_{p15}$. Eluates of spot $A_{p5}$ from two chromatograms on Whatman no. 3 paper, were hydrolysed with 12 N-HCl at 37° for 3 days and the hydrolysate was chromatographed on Whatman no. 4 paper. The results are shown in Fig. 3 and Table 2.

Eluates of spots $A_{p14}$ and $A_{p15}$ were treated with 1:2:4-fluorodinitrobenzene and the dinitrophenyl (DNP) derivatives hydrolysed 3 days with 12 N-HCl at 37°. The hydrolysates were extracted with ether, and in both cases bis-DNP-tyrosine was identified in the extract by using reversed phase paper chromatography. The aqueous solutions were chromatographed on Whatman no. 4 paper. Peptide $A_{p14}$ gave on partial hydrolysis Glu.Leu ($A_{1e8}$) Leu.Glu ($A_{1e7}$) and Glu.Leu.Glu ($A_{1\delta6}$) in addition to the free amino-acids and consequently had the structure Tyr.Glu.Leu.Glu. Peptide $A_{p15}$ gave only free amino-acids and Glu.Leu ($A_{1e8}$) on partial hydrolysis and must therefore have been Tyr.Glu.Leu.

Table 1. *Peptides from peptic hydrolysate ($A_p$) of fraction A of oxidized insulin*

| Spot no. (Figs. 1 and 2) | Amino-acids identified | Strength | DNP amino-acids identified | Structure* |
|---|---|---|---|---|
| 1 | $CySO_3H$ | × × × | | |
| | Asp | × × × × | Glu | Glu.[$CySO_3H$, Asp, Tyr] |
| | Glu | × × × | | |
| | Tyr | × × × | | |
| 2 | $CySO_3H$ | × | | |
| | Asp | × × | Glu | Glu.[$CySO_3H$, Asp, Tyr] |
| | Glu | × | | |
| | Tyr | × | | |
| 3 | $CySO_3H$ | × × × | | |
| | Asp | × × × × | Asp | Asp.[$CySO_3H$, Asp, Tyr] |
| | Tyr | × × × | | |
| 4 | $CySO_3H$ | × | | |
| | Asp | × × | Asp | Asp.[$CySO_3H$, Asp, Tyr] |
| | Tyr | × | | |
| 5 | $CySO_3H$ | × × × × × | | |
| | Glu | × × × × × | | |
| | Ser | × × × × × | | |
| | Gly | × × × × | Gly | Gly.[$CySO_3H$, Glu, Ser, Ala, Val, Leu] |
| | Ala | × × × × | | |
| | Val | × × × × × | | |
| | Leu | × × × × × | | |
| 6 | $CySO_3H$ | × × | | |
| | Asp | × × | | |
| | Glu | × × | Leu† | Leu.[$CySO_3H$, Asp, Glu, Tyr] |
| | Tyr | × × | | |
| | Leu | × × | | |
| 7 | $CySO_3H$ | × | | |
| | Asp | × × | | |
| | Glu | × | | [$CySO_3H$, Asp, Glu, Tyr, Leu] |
| | Tyr | × | | |
| | Leu | × | | |
| 8 | Glu | × | | Glutamic acid |
| 9 | $CySO_3H$ | × × | | |
| | Glu | × × | | |
| | Ser | × × | Gly | Gly.[$CySO_3H$, Glu, Ser, Ala, Val, Leu] |
| | Gly | × × | | |
| | Ala | × × | | |
| | Val | × × | | |
| | Leu | × × | | |
| 10 | Glu | × × | | [Glu, Tyr] |
| | Tyr | × × | | |
| 11 | Glu | × × | | [Glu, Tyr] |
| | Tyr | × × | | |
| 12 | Tyr | × | | Tyrosine |
| 13 | Glu | × × | | Leu.Glu |
| | Leu | × | | |
| 14 | Glu | × × × × | | |
| | Tyr | × × × | Tyr† | Tyr.[Glu, Leu] |
| | Leu | × × × | | |
| 15 | Glu | × × × | | |
| | Tyr | × × × | Tyr† | Tyr.[Glu, Leu] |
| | Leu | × × × | | |
| 16 | Glu | × | | |
| | Gly | × | | Gly.Ileu.Val.Glu ($A_{1/3}$) |
| | Val | × | | |
| | Leu | × | | |

* Where amino-acids are enclosed in square brackets the sequence has not been directly established.
† Identified by reversed-phase paper chromatography.
Spot 5. Yellow colour with ninhydrin.
Spot 9. Red-yellow colour with ninhydrin.
Spot 13. The aqueous solution of a hydrolysate of the DNP derivative contained Glu ( × $\dot{\times}$ ) Leu ( × ) suggesting it is Leu.Glu.

*Experiment $A_c$ (chymotrypsin)*

Fig. 4 shows a chromatogram of the chymotryptic hydrolysate. Since there was considerable overlapping of some of the spots, each one was purified by ionophoresis on paper in 0·05 M-ammonium acetate using the technique of Durrum (1950). With the exception of spot (6+7), only one main component was found in each case and the amino-acids given on hydrolysis are recorded in Table 3. In the case of spot (6+7) two bands were visible after ionophoresis when the paper was examined in ultraviolet light. One ($A_{p6}$) was neutral and the other ($A_{p7}$) was acidic. $A_{p6}$ gave a yellow colour with ninhydrin, whereas no colour was obtained with $A_{p7}$. The composition of the two is shown in Table 3.

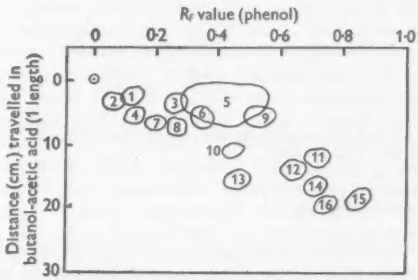


Fig. 1. Chromatogram of peptic hydrolysate of fraction $A$ of oxidized insulin (Exp. $A_p$) in which both solvents were run one length of paper (see Table 1).

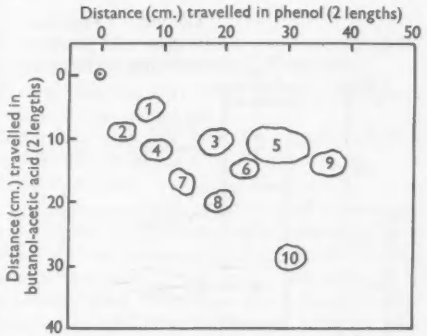


Fig. 2. Chromatogram of peptic hydrolysate of fraction $A$ of oxidized insulin (Exp. $A_p$) in which both solvents were run two lengths of paper (see Table 1).

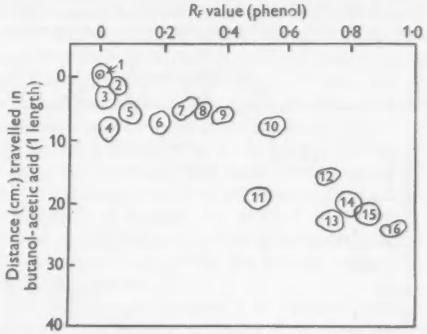


Fig. 3. Chromatogram of partial acid hydrolysate of peptide $A_{p5}$ (see Table 2).

In later experiments, when further samples of these peptides were required, it was more convenient to run a large-scale ionophoresis first, followed by two-dimensional chromatography of the bands obtained. Fig. 5 is a diagram of such an ionophoresis, showing the relative positions of the different peptides.

Table 2. *Peptides from partial hydrolysate of peptide $A_{p5}$*

| Spot no. (Fig. 3) | Strength | Structure | Identical with |
|---|---|---|---|
| 1 | × | $CySO_3H.CySO_3H.Ala$ + $Glu.CySO_3H.CySO_3H.Ala$ | $A_{2\delta1}$ + $A_{2\gamma1}$ |
| 2 | × | Cysteic acid | — |
| 3 | × | $Glu\ CySO_3H$ | $A_{1\alpha1}$ |
| 4 | × | Glu Glu | $A_{1\beta4}$ |
| 5 | × × | $CySO_3H.Ala$ | $A_{1\gamma3}$ |
| 6 | × | Glutamic acid | — |
| 7 | × × × | $Ser.Val.CySO_3H$ | $A_{1\zeta2}$ |
| 8 | × × | Serine | — |
| 9 | × × | Glycine | — |
| 10 | × × | Alanine | — |
| 11 | × × | Gly Ileu.Val.Glu.Glu. + Ileu Val Glu.Glu | $A_{1a15}$ $A_{1a16}$ |
| 12 | × | Ser.Val | $A_{1\iota6}$ |
| 13 | × | Ileu Val Glu | $A_{1\eta4}$ |
| 14 | × × | Ser Leu | $A_{1\iota7}$ |
| 15 | × | Leucine + isoleucine | — |
| 16 | ? | Ileu.Val | $A_{1\iota10}$ |

Table 3. *Peptides from chymotryptic hydrolysate ($A_c$) of fraction A of oxidized insulin*

| Spot no. (Fig. 4) | Amino-acids formed after hydrolysis: $CySO_3H$ | Asp | Glu | Ser | Gly | Ala | Tyr | Val | Leu |
|---|---|---|---|---|---|---|---|---|---|
| 1 | × × × | × × × | — | — | — | — | —. | — | — |
| 2 | × × × | — | × × × | × × × | × × × | × × × | — | × × × | × × |
| 3 | × × | × × × | × × × × | — | — | — | × × | — | × × |
| 4 | × × × × | — | × × × × | × × × × | × × × × | × × × × | × × × | × × × × | × × × × |
| 5 | — | × × | × × × | — | — | — | × × | — | × × |
| 6 | — | — | — | × × | — | — | × × × | — | × × × |
| 7 | — | × × | × × × | — | — | — | × × | — | × × |

Spot 1. Yellow colour with ninhydrin, turning to purple on prolonged heating. Hydrolysis of the DNP derivative gives DNP-cysteic acid and aspartic acids. Its structure is therefore $CySO_3H$.Asp.
Spot 2. Yellow colour with ninhydrin. Contained isoleucine but no leucine.
Spot 3. Hydrolysis of the DNP derivative gave DNP-glutamic acid.
Spot 4. Yellow colour with ninhydrin. Contained both leucine and isoleucine.

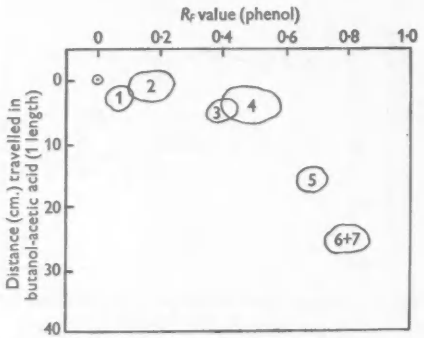


Fig. 4. Chromatogram of chymotryptic hydrolysate of fraction *A* of oxidized insulin (Exp. $A_c$) (see Table 3).

Table 4. *Action of carboxypeptidase on insulin derivatives*

| Substrate | Time of incubation at 37° | Free amino-acids liberated | Strength |
|---|---|---|---|
| Fraction *B* | 30 min. | Ala | × × × × × |
| Fraction *A* | 1 hr. | Asp (-$NH_2$) | × |
| | | Ala | ? |
| Insulin | 1·5 hr. | Ala | × × × × × |
| | | Asp (-$NH_2$) | × × |
| | | Asp | ? |
| | 8 hr. | Ala | × × × × × |
| | | Asp (-$NH_2$) | × × × |
| | | Asp | × |
| Acetylinsulin | 4 hr. | Ala | × × × × × |
| | | Asp (-$NH_2$) | × × × × |
| | | Asp | × |

*Carboxypeptidase experiments*

The results obtained with carboxypeptidase are summarized in Table 4. Free alanine was liberated very rapidly from fraction *B* and insulin, as has been shown by Lens (1949). No evidence could be found of free lysine which is the residue adjacent to alanine (Sanger & Tuppy, 1951*b*). The first amino-acids liberated from fraction *A* were asparagine and a trace of alanine, which is probably due to contamination with fraction *B*. On prolonged incubation traces of glutamic acid, serine, tyrosine and leucine were liberated. Better yields of asparagine were obtained from insulin. This effect may be due to inhibition of the enzyme by the —$SO_3H$ group of the adjacent cysteic acid residue in fraction *A*. An approximate estimation by the spot-dilution technique of Polson, Mosley & Wyckoff (1947) indicated

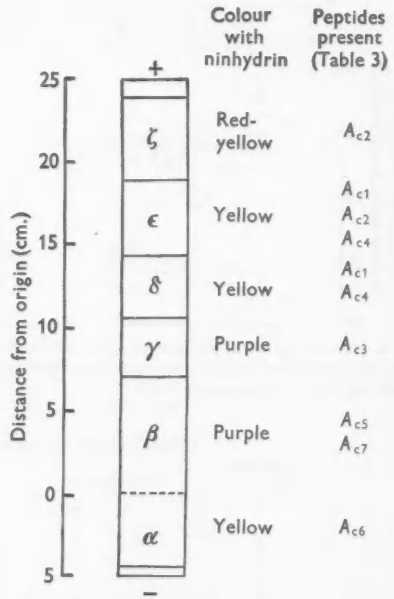


Fig. 5. Ionophoresis of chymotryptic hydrolysate of fraction *A* of oxidized insulin in 0·05 M-ammonium acetate (see Table 3).

that after 8 hr. incubation the yield of asparagine from insulin was 25–30% of the theoretical value, assuming two *C*-terminal asparagine residues per molecule of mol.wt. 12000.

Acetylinsulin was investigated in the hope that the lysine residue from the phenylalanyl chain could be split off as ε-acetyllysine. This expectation was not realized, but a much better yield of asparagine was obtained. After a reaction period of 4 hr. the yields of both alanine and asparagine were approximately 90% of the theoretical value. Aspartic acid was also obtained in a yield of less than 10%. It is probably a secondary product derived from asparagine, although control experiments in which asparagine was incubated with carboxypeptidase showed that the breakdown was very small and would hardly account for the amounts of aspartic acid formed in the experiments described above.

## DISCUSSION

### *Amino-acid sequence of fraction A*

In the previous paper four sequences were deduced as being present in fraction *A*. These were:

(1) Gly.Ileu.Val.Glu.Glu.$CySO_3H$.$CySO_3H$.Ala (*N*-terminal sequence).
(2) Ser.Leu.Tyr.Glu.Leu.Glu.Asp.Tyr.$CySO_3H$.
(3) Ser.Val.$CySO_3H$.
(4) $CySO_3H$.Asp.

The peptide present in the highest concentration in the peptic hydrolysate was peptide $A_{ps}$. It contained all the amino-acids of fraction *A* except aspartic acid and tyrosine, and had a glycine *N*-terminal residue. On partial hydrolysis with acid it gave the peptides listed in Table 2. Of these, all but peptides 7 (Ser.Val.$CySO_3H$), 12 (Ser.Val) and 14 (Ser.Leu) are derived from sequence 1. Since peptide $A_{ps}$ gives Ser.Leu on hydrolysis but contains no tyrosine, this leucine residue must be present as the *C*-terminal residue. It contains therefore sequence 1 as its *N*-terminal sequence, Ser.Leu as its *C*-terminal sequence and Ser.Val.-$CySO_3H$ which must be within the chain. Neither $CySO_3H$.Asp nor the remainder of sequence 2 can be present in $A_{ps}$, so that its structure must be Gly.Ileu.Val.Glu.Glu.$CySO_3H$.$CySO_3H$.Ala.-Ser.Val.$CySO_3H$.Ser.Leu. Having established this sequence containing three cysteic acid residues there is only one such residue to which $CySO_3H$.Asp can be attached and the structure of fraction *A* must be Gly.Ileu.Val.Glu.Glu.$CySO_3H$.$CySO_3H$.-Ala.Ser.Val.$CySO_3H$.Ser.Leu.Tyr.Glu.Leu.-Glu.Asp.Tyr.$CySO_3H$.Asp.

For simplicity in deducing this sequence, considerable use has been made of the figures given in the previous paper (Sanger & Thompson, 1953*a*, Table 13) for the amino-acid composition of fraction *A*. It could, however, have been deduced as the simplest structure which would fit all the experimental results obtained, and, in fact, was originally worked out in this way. For instance, it could be shown that all the tyrosine-containing peptides could not be explained on the basis of one sequence, but could be explained on the basis of two sequences. This is fairly conclusive evidence that there are two tyrosine residues. If there were three it is unlikely that all the peptides could be fitted into two sequences. While the structure deduced is the simplest one that will fit the experimental results, a few more complicated ones are possible but are ruled out by the analytical figures. For instance, if there were an extra serine residue between positions 8 and 9 (Table 5) this might not have been detected, but the analytical results show that there are only two such residues in the chain. It is seen that an exact amino-acid analysis of the chain may in this way be worked out from the nature of the peptides without carrying out any quantitative estimation. It is interesting that the very approximate analysis of fraction *A* (Table 13, Sanger & Thompson, 1953*a*) which was done by paper chromatography is in fact correct except for the leucine value which should be 2 and not 3.

The fact that it is possible to deduce this single sequence proves that fraction *A* is an essentially homogeneous peptide and therefore that there is only one type of glycyl chain in insulin.

As was the case with the phenylalanyl chain, there is no obvious periodicity of the amino-acids in this chain. In fraction *B* it was noted that the aromatic and less polar residues were grouped together. There is no such arrangement in fraction *A*, so that this cannot be regarded as a general property of proteins. In fraction *B* there were three dipeptide sequences which occurred twice in the chain, and this was considerably more than would be expected on a random arrangement. No dipeptide sequences occur twice in the glycyl chain, although there are now six which occur twice in the whole insulin. These are Val.Glu (*A* 3–4, *B* 12–13), Val.$CySO_3H$ (*A* 10–11, *B* 18–19), Leu.Tyr (*A* 13–14, *B* 15–16), His.Leu (*B* 5–6, *B* 10–11), Gly.$CySO_3H$ (*B* 7–8, *B* 19–20) and Leu.Val (*B* 11–12, *B* 17–18). The sequence CyS.Gly, which occurs in glutathione and twice in the phenylalanyl chain, is not found in the glycyl chain. It would thus seem that no general conclusions can be drawn from these results concerning the general principles which govern the arrangement of amino-acid residues in protein chains. In fact, it would seem more probable that there are no such principles, but that each protein has its own unique arrangement; an arrangement which endows it with its particular properties and specificities and fits it for the function that it performs in nature.

Table 5. *Peptides obtained by enzymic hydrolysis of fraction A of oxidized insulin*

| | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sequences deduced from lower peptides (Sanger & Thompson, 1953*a*) | Gly. | Ileu. | Val. | Glu. | Glu. | $CySO_3H$. | $CySO_3H$. | Ala | | | | | | Ser. | Leu. | Tyr. | Glu. | Leu. | Glu. | Asp. | Tyr. | $CySO_3H$ |
| | | | | | | | | | | Ser. | Val. | $CySO_3H$ | | | | | | | | | | $CySO_3H$. | Asp |
| Peptides from peptic hydrolysate | Gly. | Ileu. | Val. | Glu. | Glu. | $CySO_3H$. | $CySO_3H$. | Ala. | Ser. | Val. | $CySO_3H$. | Ser. | Leu | | | | Leu. | Glu. | Asp. | Tyr. | $CySO_3H$. | Asp |
| | | | | | | | ($A_{p5}$) | | | | | | | | | | | | ($A_{p6}$, $A_{p7}$) | | | |
| | Gly. | Ileu. | Val. | Glu. | Glu. | $CySO_3H$. | $CySO_3H$. | Ala. | Ser. | Val | | | | Tyr. | Glu | | Glu. | Asp. | Tyr. | $CySO_3H$. | Asp | |
| | | | | | | ($A_{p9}$) | | | | | | | | ($A_{p10}$, $A_{p11}$) | | | ($A_{p1}$, $A_{p8}$) | | | | | |
| | Gly. | Ileu. | Val. | Glu | | | | | | | | | | Tyr. | Glu. | Leu | | Asp. | Tyr. | $CySO_3H$. | Asp | |
| | ($A_{p16}$) | | | | | | | | | | | | | ($A_{p15}$) | | | | ($A_{p2}$, $A_{p4}$) | | | | |
| | | | | | | | | | | | | | | Tyr. | Glu. | Leu. | Glu | | | | | |
| | | | | | | | | | | | | | | ($A_{p14}$) | | | | | | | | |
| | | | | | | | | | | | | | | | | Leu. | Glu | | | | | |
| | | | | | | | | | | | | | | | | ($A_{p13}$) | | | | | | |
| Peptides from chymotryptic hydrolysate | Gly. | Ileu. | Val. | Glu. | Glu. | $CySO_3H$. | $CySO_3H$. | Ala. | Ser. | Val. | $CySO_3H$. | Ser. | Leu. | Tyr | | | | | | | $CySO_3H$. | Asp |
| | | | | | | | ($A_{c4}$) | | | | | | | | | | | | | | ($A_{c1}$) | |
| | Gly. | Ileu. | Val. | Glu. | Glu. | $CySO_3H$. | $CySO_3H$. | Ala. | Ser. | Val. | $CySO_3H$ | | | | | | Glu. | Leu. | Glu. | Asp. | Tyr. | $CySO_3H$. | Asp |
| | | | | | | ($A_{c2}$) | | | | | | | | | | | | | | ($A_{c3}$) | | | |
| | | | | | | | | | | | | Ser. | Leu. | Tyr | | | | | | | | | |
| | | | | | | | | | | | | ($A_{c6}$) | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | Glu. | Leu. | Glu. | Asp. | Tyr | | |
| | | | | | | | | | | | | | | | | | ($A_{c5}$, $A_{c7}$) | | | | | | |
| Structure of fraction *A* | Gly. | Ileu. | Val. | Glu. | Glu. | $CySO_3H$. | $CySO_3H$. | Ala. | Ser. | Val. | $CySO_3H$. | Ser. | Leu. | Tyr. | Glu. | Leu. | Glu. | Asp. | Tyr. | $CySO_3H$. | Asp |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | |
| Bonds split by pepsin | | | | ↑ | | | | | | ↑ | | | | ↑ | ↑ | ↑ | ↑ | ↑ | | | | |
| Bonds split by chymotrypsin | | | | | | | | | | | ↑ | | | ↑ | | | | | ↑ | | | |

↑, major sites of action of enzymes; ↑, other bonds split by enzymes.

*Structure of the peptides*

Having established the sequence of residues in fraction *A* it is now possible to consider the peptides identified in the various hydrolysates. Their structures are given in Table 5. The fact that these can all be determined in this way provides confirmatory evidence for the main sequence.

Since pepsin splits bonds involving the amino groups of tyrosine residues in synthetic peptides (Fruton & Bergmann, 1939), it was expected that splitting of fraction *A* would occur at position 18–19 to give Tyr.$CySO_3H$.Asp. Although peptides containing these three amino-acids were present ($A_{p3}$, $A_{p4}$), they had aspartic acid and not tyrosine as the *N*-terminal residues. Splitting by pepsin therefore occurred at bond 17–18 (Glu.Asp) and no evidence could be obtained for splitting of bond 18–19. Further considerations on the specificity of pepsin will be presented in a later paper (Sanger & Thompson, 1953*b*), when the positions of the amide groups have been established.

The presence of duplicate spots of the peptides Asp.Tyr.$CySO_3H$.Asp ($A_{p3}$, $A_{p4}$), Glu.Asp.Tyr.-$CySO_3H$.Asp ($A_{p1}$, $A_{p2}$) and Leu.Asp.Tyr.-$CySO_3H$.Asp ($A_{p6}$, $A_{p7}$) is probably due to deamidation. Amide groups are present on the aspartic acid residues (Sanger & Thompson, 1953*b*) and some hydrolysis probably occurred during the digestion with pepsin in acid solution (cf. Melville, 1935). Similarly, peptides $A_{p10}$ and $A_{p11}$ (Tyr.Glu) differ by an amide group.

Partial acid hydrolysis of peptide $A_{p9}$ on a rather small amount of material suggested the structure shown in Table 5, since it gave the peptides from the *N*-terminal sequence and Ser.Val but no Ser.Val.-$CySO_3H$ or Ser.Leu.

In the chymotryptic hydrolysate two peptides ($A_{c5}$, $A_{c7}$) containing the same amino-acids (Asp, Glu, Tyr, Leu) are present. $A_{c7}$ moves faster on the chromatograms than $A_{c5}$ and gives no colour with ninhydrin, suggesting that it has no free α-amino group. Both are derived from the sequence Glu.Leu.Glu.Asp.Tyr and evidence to be presented later (Sanger & Thompson, 1953*b*) indicates that this *N*-terminal glutamic acid residue is present in the form of an amide. Glutamine residues containing a free α-amino group are particularly labile and readily cyclize to give the corresponding pyrollidonecarboxylic acid derivative (Melville, 1935). It seems probable that peptide $A_{c7}$ is such a derivative formed from $A_{c5}$ during the digestion or subsequent operations.

Peptide $A_{c6}$ can only be Ser.Leu.Tyr identical with $A_{1a19}$, indicating that splitting with chymotrypsin has occurred at bond 11–12 ($CySO_3H$.Ser). This is confirmed by the presence of peptide $A_{c2}$ which contains isoleucine and the other amino-acids from the *N*-terminal sequence but no leucine. Since the splitting of this bond was rather unexpected, control experiments were carried out to show that the effect was due to chymotrypsin and not to the experimental conditions used. An end-group determination on the fraction *A* used showed that there were no free amino groups of serine present, glycine being the only *N*-terminal residue. Samples of fraction *A* were then treated in the usual manner with chymotrypsin which had been previously incubated at 37° for 30 min. with a 0·001 M solution of the specific inhibitor di*iso*propylfluorophosphonate. Chromatography of the evaporated solutions showed no spot corresponding to peptide $A_{c2}$. Only one large area ($R_F$ in phenol = 0·25) was obtained on the chromatograms which gave a yellow colour with ninhydrin and on hydrolysis gave all the amino-acids of fraction *A*. It was obviously unchanged fraction *A*. It is therefore concluded that the splitting of this bond is due to the action of chymotrypsin or possibly of another enzyme that is very closely associated with it and which is inhibited by di*iso*propyl fluorophosphonate.

Further evidence for this splitting was provided in an experiment in which peptide $A_{p5}$ was treated with chymotrypsin. Ser.Leu was identified as the only neutral component after ionophoresis in 0·05 M-ammonium acetate. The bands moving towards the anode were separated by chromatography and shown to be similar in $R_F$ values and composition to $A_{c2}$ and $A_{p5}$ (unchanged peptide).

*The C-terminal residue of fraction A*

Peptide $A_{c1}$ contains the same amino-acid sequence as peptide $A_{1a2}$ ($CySO_3H$.Asp). However, it was found to behave differently on ionophoresis in 0·05 M-ammonium acetate. Thus after ionophoresis for 6 hr. with a potential of 220 V., $A_{1a2}$ had moved 8·4 cm., whereas $A_{c1}$ had only moved 5·1 cm. This clearly shows that $A_{c1}$ is an amide. Its structure is therefore $CySO_3H$.Asp(-$NH_2$) and the *C*-terminal residue of fraction *A* is asparagine. Although there is now considerable confirmatory evidence for this (Chibnall & Rees, 1952; Harris, 1952), it had been suggested at the time this sequence was first being worked out, that glycine was present as a *C*-terminal residue in insulin, presumably on the glycyl chains (Fromageot, Jutisz, Meyer & Penasse, 1950; Chibnall & Rees, 1951). As a result of more recent work Chibnall & Rees (private communication) have now withdrawn this claim.

The analytical results on fraction *A* indicated that there was only one glycine residue and this appeared to be the *N*-terminal residue. This was supported by the results of Butler, Phillips, Stephen & Creeth (1950), which we have been able to confirm. They showed that no glycine was present in

a hydrolysate of the DNP derivative of fraction *A*, showing that the only glycine residues are *N*-terminal.

The results with carboxypeptidase support the conclusion that asparagine is the *C*-terminal residue of the glycyl chain. In the case of acetylinsulin the liberation was approximately quantitative, assuming that it is present in both the glycyl chains, if one assumes that the molecular weight is 12000.

## SUMMARY

1. Fraction *A* of oxidized insulin was hydrolysed with pepsin and chymotrypsin. The resulting peptides were fractionated and their structure investigated.

2. It is concluded that the amino-acid sequence in the glycyl chain of insulin is Gly . Ileu . Val . Glu . Glu . CyS . CyS . Ala . Ser . Val . CyS . Ser . Leu . Tyr . Glu . Leu . Glu . Asp . Tyr . CyS . Asp.

We wish to express our thanks to Mr J. Lightbown for the chymotrypsin and to Dr B. C. Saunders for the di*iso*propyl fluorophosphonate.

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# The Branched-chain Fatty Acids of Mutton Fat

## 2. THE ISOLATION OF (+)-12-METHYLTETRADECANOIC ACID AND OF 13-METHYLTETRADECANOIC ACID

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Following the isolation of branched-chain fatty acids from butterfat (Hansen & Shorland, 1950, 1951, 1952; Hansen, Shorland & Cooke, 1951) and from ox fat (Hansen, Shorland & Cooke, 1952*a*), a detailed examination was made of the lipids extracted from the external fatty tissue of old ewes. The occurrence of (+)-14-methylhexadecanoic acid as a constituent of this fat was recently established (Hansen, Shorland & Cooke, 1952*b*, *c*). In this present paper are reported the isolation from mutton fat of two isomeric $C_{15}$ branched-chain saturated fatty acids, (+)-12-methyltetradecanoic acid and 13-methyltetradecanoic acid.

Although the optically active *ante-iso*-acid, (+)-12-methyltetradecanoic acid, has been isolated from wool grease (Weitkamp, 1945), the *iso* acid, 13-methyltetradecanoic acid, has not formerly been found in natural fats.

## EXPERIMENTAL

The long crystal spacings reported in this work were determined by means of a Philips Geiger X-ray spectrometer. Except where otherwise indicated, nickel-filtered copper $K\alpha$ radiation was used. Samples were melted on a glass slide and quickly cooled.

All melting points were determined in closed capillaries and are uncorrected.

Analyses were by Weiler and Strauss, Oxford.

The fat used in this investigation was obtained by mincing and then steam-rendering the external fatty tissues

of old overweight ewes (Hansen *et al.* 1952*c*). The extracted glycerides (sap. equiv. 286·9, iodine value 46·6, unsaponifiable matter 0·56 %, free fatty acids 0·4 %) were converted to methyl esters (7·69 kg.) and hydrogenated at 180°, Ni supported on kieselguhr being used as a catalyst. The hydrogenated methyl esters (iodine value 3·0) were then repeatedly crystallized from 10 vol. acetone at −30° and finally yielded 332·6 g. of 'liquid' methyl esters (iodine value 7·0). Fractional distillation at approx. 0·2 mm. in a 22-plate Vigreux column provided a series of thirteen fractions and a residue. (See Table 1, Hansen *et al.* 1952*c*.) Fraction HL6 of this series (wt. 21·48 g., sap. equiv. 256·0, iodine value 1·1) was then refractionated at approx. 0·1 mm. in a 60 cm. electrically heated and packed column which possessed about twenty-one plates (Table 1). As fractions HL6L5, HL6L6 and HL6L7 had similar molecular weights, they were bulked together (12·0 g.) and denoted H2. Repeated low-temperature crystallization of H2 esters from different solvents effected separation into three main fractions as shown in Table 2: H2SL2S2L2L (1·55 g., m.p. −11·8°, sap. equiv. 257·6, iodine value nil); H2SL2S4L (1·04 g., m.p. −0·2°); and H2SL2S4S (0·67 g., m.p. 1·5°).

Table 1. *Fractional distillation of HL6 methyl esters*

(Pressure 0·1 mm.)

| Fraction no. | Wt. (g.) | Sap. equiv. |
|---|---|---|
| HL6L1 | 0·96 | 247·0 |
| HL6L2 | 1·23 | 245·3 |
| HL6L3 | 2·77 | 249·1 |
| HL6L4 | 3·07 | 252·7 |
| HL6L5 | 7·53 | 258·0 |
| HL6L6 | 2·18 | 258·5 |
| HL6L7 | 2·64 | 259·8 |
| HL6LR | 0·87 | 278·7 |

Fraction H2SL2S2L2L was converted to fatty acids and denoted H25. Repeated low-temperature fractional crystallization of H25 acids from ether and from methanol yielded fraction H25S2LS (Table 3).

Further purification was effected by eluting the methyl esters of this fraction (renamed H35) with light petroleum (b.p. 50–60°) through an $Al_2O_3$ column (see Table 4).

Table 4. *Chromatographic separation of the methyl esters of fraction H35*

(Eluant, light petroleum, b.p. 50–60°)

| Fraction no. | Vol. of eluant (ml.) | Wt. (g.) | $n_D^{20°}$ | Sap. equiv. (methyl ester) |
|---|---|---|---|---|
| H35a | 25 | 0·23 | 1·4385 | 249·2 |
| b | 25 | 0·30 | 1·4383 | 254·0 |
| c | 25 | 0·09 | 1·4377 | — |
| d | 25 | 0·03 | 1·4377 | — |
| e | 50 | 0·02 | 1·4385 | — |
| f | 75 | Trace | — | — |

Fraction H35b (wt. 0·30 g., sap. equiv. 254·0, m.p. 27·0 (acid) X-ray long spacing 30·1 A. (acid), $[\alpha]_D^{25°}$ +3·7° (acid) in $CHCl_3$) was then submitted in the form of acids to low-temperature crystallization from methanol and from acetone (Table 5), H35bL2S finally separating out as a white crystalline substance: wt. 0·11 g., m.p. 25·8°, sap. equiv. 241·6, iodine value (Wijs) 1·1. The chemical and physical characteristics of fractions H35b and H35bL2S are shown in Table 6.

Table 2. *Low-temperature crystallization of methyl esters (H2) (12·0 g.)*

| | | Soluble | | | Insoluble | | |
|---|---|---|---|---|---|---|---|
| Fraction | Conditions of crystallization: solvent; temp. (°) | Fraction | Wt. (g.) | M.p. (°) | Fraction | Wt. (g.) | M.p. (°) |
| H2 | Ether, 4 vol. | −68 | | | | | |
| H2S | Light petroleum, 40 vol. | −70 | H2SL | 8·24 | −13 | H2SS | 2·52 | −14 |
| H2SL | Ether, 40 vol. | −70 | H2SLL | 8·08 | — | H2SLS | 0·11 | — |
| H2SLL | Acetone, 20 vol. | −70 | H2SL2L | 2·53 | — | H2SL2S | 5·52 | — |
| H2SL2S | Acetone, 20 vol. | −60 | H2SL2SL | 0·57 | — | H2SL2SS | 4·87 | — |
| H2SL2SS | Acetone, 20 vol. | −50 | H2SL2S2L | 1·65 | — | H2SL2S2S | 3·21 | — |
| H2SL2S2L | Ether, 20 vol. | −55 | H2SL2S2LL | 1·61 | — | H2SL2S2LS | 0·03 | — |
| H2SL2S2LL | Ether, 40 vol. | −70 | H2SL2S2L2L (Sap. equiv. 257·6; iodine value Nil) (Converted to acids, H25) | 1·55 | −11·8 | H2SL2S2L2S | 0·04 | — |
| H2SL2S2S | Acetone, 20 vol. | −40 | H2SL2S3L | 1·44 | — | H2SL2S3S | 1·76 | — |
| H2SL2S3S | Acetone, 20 vol. | −30 | H2SL2S4L (Converted to acids, H28) | 1·04 | −0·2 | H2SL2S4S (Converted to acids, H29) | 0·67 | 1·5 |

Table 3. *Low-temperature crystallization of fatty acids (H25) (1·39 g.)*

| | | Soluble | | | Insoluble | | |
|---|---|---|---|---|---|---|---|
| Fraction | Conditions of crystallization: solvent; temp. (°) | Fraction | Wt. (g.) | M.p. (°) | Fraction | Wt. (g.) | M.p. (°) |
| H25 | Ether, 10 vol. | −70 | H25L | 0·10 | 15·0 | H25S | 1·28 | 26·8 |
| H25S | Ether, 10 vol. | −65 | H25SL | 0·15 | 23·2 | H25SS | 1·12 | 26·0 |
| H25SS | Methanol, 100 vol. | −40 | H25S2L | 1·04 | 26·5 | H25S2S | 0·08 | 27·2 |
| H25S2L | Methanol, 40 vol. | −40 | H25S2LL | 0·29 | 24·0 | H25S2LS (Converted to methyl esters, H35) | 0·74 | 27·5 |

Table 5. *Purification of fatty acid fraction H35b* (0·21 *g., m.p.* 27·0°)

| | | Soluble | | | Insoluble | | |
|---|---|---|---|---|---|---|---|
| Fraction | Conditions of crystallization: solvent; temp. (°) | Fraction | Wt. (g.) | M.p. (°) | Fraction | Wt. (g.) | M.p. (°) |
| H35b | Methanol, 100 vol. −30 | H35bL | 0·20 | 26·8 | H35bS | 0·01 | — |
| H35bL | Methanol, 100 vol. −35 | H35bLL | 0·17 | 26·5 | H35bLS | 0·02 | 27·2 |
| H35bLL | Acetone, 20 vol. −40 | H35bL2L | 0·05 | 24·0 | H35bL2S (Sap. equiv. 241·6) | 0·11 | 25·8 |

Table 6

(*a*) *Characteristics of fatty acid fraction H35bL2S and of H35b from which it was isolated*

| Fraction no. | Wt. (g.) | M.p. (°) | Sap. equiv. | Combustion analysis (%) | Iodine value (Wijs) | *C*-Methyl | X-ray long spacing | Specific rotation |
|---|---|---|---|---|---|---|---|---|
| H35b | 0·21 | 27·0 | 254·0 (Me ester) | — | — | — | 30·1 A. | $[\alpha]_D^{22°}$ +3·7° ($CHCl_3$) |
| H35bL2S | 0·11 | 25·8 | 241·6 | C, 74·7 H, 12·3 | 1·1 | 8·38% (equiv. to 1·35 mols. acetic acid) | — | — |
| Calc. for $C_{15}H_{30}O_2$: | | | 242·4 | C, 74·4 H, 12·5 | | | | |

(*b*) *Characteristics of fatty acid fraction H29S2L2S*

| Fraction no. | Wt. (g.) | M.p. (°) | Sap. equiv. | Combustion analysis (%) | Iodine value (Wijs) | *C*-Methyl | X-ray long spacing |
|---|---|---|---|---|---|---|---|
| H29S2L2S | 0·25 | 50·2 | 241·7 | C, 74·4 H, 12·4 | 0·9 | 10·7% (equiv. to 1·72 mols. acetic acid) | 30·05 A. |
| Theor. for $C_{15}H_{30}O_2$: | | | 242·4 | C, 74·4 H, 12·5 | | | |

Table 7. *Low-temperature crystallization of fatty acids* (*H29*) (0·60 *g.*)

| | | Soluble | | | Insoluble | | |
|---|---|---|---|---|---|---|---|
| Fraction | Conditions of crystallization: solvent; temp. (°) | Fraction | Wt. (g.) | M.p. (°) | Fraction | Wt. (g.) | M.p. (°) |
| H29 | Ether, 40 vol. −50 | H29L | 0·23 | 45·0 | H29S | 0·37 | 50·0 |
| H29S | Ether, 40 vol. −50 | H29SL | 0·06 | 45·2 | H29SS | 0·31 | 50·2 |
| H29SS | Methanol, 100 vol. room temp. | H29S2L | 0·30 | — | H29S2S | 0·01 | — |
| H29S2L | Methanol, 100 vol. −7 | H29S2LL | 0·28 | 50·0 | H29S2LS | 0·02 | 49·2 |
| H29S2LL | Methanol, 40 vol. −40 | H29S2L2L | 0·02 | 42·0 | H29S2L2S (Sap. equiv. 241·7) | 0·25 | 50·2 |

Subsequent to the isolation of H35bL2S four further fractions with properties corresponding to those of H35bL2S were independently extracted by means of fractional distillation and repeated crystallization from bulked fractions HL7, HL8 and HL9. (See Table 1, Hansen *et al.* 1952*c*.) The characteristics of these four fractions were as follows:

H51S4LS2S, wt. 0·25 g., m.p. 26·5°, sap. equiv. 244·3, X-ray long spacing 29·97 A. (Fe $K\alpha$).

H63L2S, wt. 0·19 g., m.p. 26·2°, sap. equiv. 240·5, X-ray long spacing 29·97 A. (Fe $K\alpha$).

H52S3LSS, wt. 0·52 g., m.p. 25·0°, sap. equiv. 244·1.

H52S4LS, wt. 0·90 g., m.p. 25·5°, sap. equiv. 241·0, $[\alpha]_D^{16°}$ +3·9° ($CHCl_3$).

By means of low-temperature crystallization of fraction H2SL2S4S (renamed H29) from ethyl ether and from methanol (see Table 7), fraction H29S2L2S was isolated: 0·25 g., m.p. 50·2°, sap. equiv. 241·7. The chemical and physical characteristics of fraction H29S2L2S are recorded in Table 6*b*.

When a further acid fraction, H2SL2S4L (Table 2), was independently submitted to a series of low-temperature crystallizations similar to those employed for H2SL2S4S, it yielded a final product H28S5LS with the following properties: wt. 0·21 g., m.p. 51·2°, sap. equiv. 242·5; *C*-Me, 10·0; C, 74·7; H, 12·4; iodine value 8·7; X-ray long spacing 30·07 A. Both H28S5LS and H29S2L2S had common origin in fraction H2SL2S2S (Table 2). A third fraction, H67SLSLSS, with properties corresponding closely with those of H29S2L2S, was derived from bulked fractions HL7, HL8 and HL9 (Table 1, Hansen *et al.* 1952*c*). Purification of this fraction was effected by distillation (0·1 mm.) in a 29-plate column (column *C*; Shorland, 1952) followed by selective bulking of fractions, redistillation (0·05 mm.) in a

30-plate column (column *E*; Shorland, 1952), and repeated low-temperature fractional crystallization. Properties of H67SLSLSS are as follows: wt. 0·56 g., m.p. 50·5°, sap. equiv. 240·4.

## DISCUSSION

The two fractions H35bL2S and H29S2L2S isolated from the external body fat of ewes are both characterized by an absence of appreciable iodine value and both possess saponification equivalents and combustion analyses in close accord with the requirements for a saturated fatty acid with the empirical formula $C_{15}H_{30}O_2$ (see Table 6). The *C*-methyl values represent the liberation during chromic acid oxidation, by the Kuhn-Roth (1933) method, of acetic acid in amounts equivalent to 1·35 and 1·72 mols. respectively, and, when interpreted according to the evidence of Ginger (1944), indicate the presence of one side-chain methyl group in each substance. X-ray diffraction analyses indicate long spacings of 30·1 and 30·05A. for fractions H35bL2S and H29S2L2S respectively.

These values are in close agreement with recorded spacings of 29·2A. for the amide derived from 12-methyltetradecanoic acid (Velick, 1947) and of 29·8A. for 13-methyltetradecanoic acid (Arosenius, Ställberg, Stenhagen & Tagtström-Eketorp, 1949). *n*-Pentadecanoic acid, by contrast, has an X-ray long spacing of 35·75A. (Slagle & Ott, 1933).

The melting point of H35bL2S further distinguishes it from *n*-pentadecanoic acid which has been accredited with melting points of 52·4° (Weitkamp, 1945) and 52·26° (Meyer & Reid, 1933). A melting point of 25·8° for H35bL2S is, however, consistent with this substance being a $C_{15}$ *ante-iso*-acid (branching on the antepenultimate carbon) the value approximating that of 23·0° obtained by Weitkamp (1945) for the 12-methyltetradecanoic acid isolated from wool grease.

The optical rotation of fraction H35bL2S, $[\alpha]_D^{22°} + 3·7°$ (in chloroform), further correlates it with Weitkamp's (1945) (+)-12-methyltetradecanoic acid $[\alpha]_D^{26°} + 4·7°$ (pure substance).

The melting point of 50·2° for H29S2L2S agrees closely with that of 51·7–51·8° for the $C_{15}$ *iso*-acid synthesized by Arosenius *et al.* (1949), and conforms approximately to the observation of Weitkamp (1945) and of Cason & Winans (1950) that *iso*-acids melt less than 1° below the melting point of the normal isomer. From the evidence presented above the two saturated branched-chain acids reported in this paper are identified as (+)-12-methyltetradecanoic acid (H35bL2S) and 13-methyltetradecanoic acid (H29S2L2S).

13-Methyltetradecanoic acid is an example of a saturated branched-chain *iso*-acid with an odd number of carbon atoms. All the *iso*-acids located in wool wax (Weitkamp, 1945) possessed an even number of carbon atoms, but an *iso*-acid with an odd number of carbons has previously been isolated from butterfat (Hansen & Shorland, 1951).

Although the total content of (+)-12-methyltetradecanoic acid has not been determined, those fractions already investigated indicate it to be present in quantities greater than 0·04% of the total weight of fatty acids. Only minor amounts of 13-methyltetradecanoic acid (about 0·02% of the total fatty acids) have been isolated, but as the *iso*-acids have melting points and solubilities similar to those of normal acids, it is to be expected that they will be present in greater amount in the 'solids', which were separated by fractional crystallization from acetone at −30°, and which have not as yet been investigated in detail.

From the fatty acids of the coccygeal glands of ducks (Weitzel & Lennert, 1951; Weitzel, 1951) have been isolated an optically active methyl-branched $C_7$ saturated acid, 4-methylhexanoic acid (contaminated with traces of 2-methylhexanoic acid), together with appreciable amounts of other unidentified alkyl-substituted acids. As the total lipids extracted from these glands contained 47·6% fatty acids, 48·0% octadecanol, and only 1·5% glycerol, the authors concluded that the fatty substances of these glands consist in the main of esters of octadecyl alcohol. Similarly, the branched-chain acids of wool wax are combined mainly with sterols and triterpenoids (Truter, 1951), and those of bacteria appear to be associated not with glycerol, as in true fats, but with some water-soluble compound such as the disaccharide trehalose (Anderson, 1941). As the extracted sample of mutton fat analysed in this present investigation contained only 0·56% unsaponifiable matter, and as there is no evidence to suggest the presence of alcohols other than glycerol, it appears that these branched-chain fatty acids are present in the fatty tissue as glycerides.

## SUMMARY

From the external fatty tissue of sheep two $C_{15}$ branched-chain saturated fatty acids, (+)-12-methyltetradecanoic acid and 13-methyltetradecanoic acid have been isolated.

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## ADDENDUM

# The Stereochemistry of *ante-iso*-Acids


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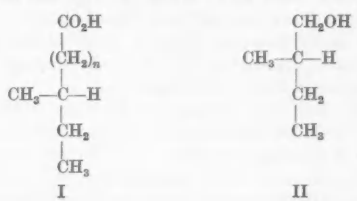
The $C_{15}$, $C_{17}$ and $C_{19}$ *ante-iso*-acids isolated from mutton-fat (Hansen, Shorland & Cooke, 1952, 1953) and wool fat (Weitkamp, 1945) are all dextrorotatory (Table 1) and the Distance Rule (Entfernungssatz) of Tschugaeff (1898) shows that all of them must have the same configuration at the asymmetric carbon atom. The following evidence indicates that they are almost certainly L-methylalkanoic acids, using the term 'L-methyl' to denote a methyl group on the left-hand side of the chain in a Fischer projection (cf. formula I) following the proposals of Ställberg-Stenhagen (1949) and of Klyne (1951).

The synthesis of (+)-14-methylhexadecanoic acid by Velick & English (1945) correlates its configuration with that of (−)-2-methylbutan-1-ol. Crombie & Harper (1950) have shown that (+)-6-methyloctanoic acid is configurationally related to (−)-2-methylbutanol (II). They point out that the correlation of (−)-2-methylbutanol with L-(−)-glyceraldehyde (Fredga, 1941, 1942, 1944) is rather involved, but it seems sound and is becoming generally accepted (cf. Birch, 1950).




I II

Table 1. *Molecular rotations of* (+)-ante-iso-*acids*

| | Formula I ($n$=) | $[M]_D$ | Reference |
|---|---|---|---|
| 2L-Methylbutanoic acid | 0 | +18 | a |
| 3L-Methylpentanoic acid | 1 | +10 | b |
| 4L-Methylhexanoic acid | 2 | +11 | c |
| 6L-Methyloctanoic acid | 4 | +13 | d |
| 12L-Methyltetradecanoic acid | 10 | +10 | e, f |
| 14L-Methylhexadecanoic acid | 12 | +14<br>+14, +12 | e, g, h |
| 16L-Methyloctadecanoic acid | 14 | +12 | e |
| 21L-Methyltricosanoic acid | 19 | +13 | i |

a, Marckwald (1899); b, Marckwald & Nolda (1909); c, Welt (1894); d, Crombie & Harper (1950); e, Weitkamp (1945); f, Hansen *et al.* (1953); g, Velick & English (1945); h, Hansen *et al.* (1952); i, Ställberg-Stenhagen (1950).

Ställberg-Stenhagen (1950) has synthesized (−)-21-methyltricosanoic acid ($[M]_D$, −13) by a route which shows that its methyl group is D-oriented in terms of the Fischer convention. The (+)-acid would therefore have $[M]_D$ +13, and would be the 21 L-methyl acid.

The (+)-*ante-iso* acids, the rotations of which are collected in Table 1, are therefore L-methyl acids as shown by formula I.


## SUMMARY

The (+)-*ante-iso*-alkanoic acids are represented by the Fischer projection formula I, and may be termed L-methyl-substituted acids.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# The Relation of Liver Kynureninase to Tryptophan Metabolism in Pyridoxine Deficiency


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The enzyme kynureninase splits alanine from the side chain of kynurenine and hydroxykynurenine to form anthranilic and hydroxyanthranilic acids, respectively (Kotake & Nakayama, 1941; Braunshtein & Goryochenkova, 1949; Wiss & Fuchs, 1950). The enzyme requires pyridoxal phosphate as coenzyme, and its activity in liver is decreased in pyridoxine deficiency (Braunshtein & Goryochenkova, 1949; Dalgliesh, Knox & Neuberger, 1951; Mason & Berg, 1952). The substrates of this enzyme, kynurenine and hydroxykynurenine, and their derivatives which retain the original carbon skeleton of the tryptophan side chain, accumulate in pyridoxine-deficient animals (Dalgliesh, 1951; 1952*a*). Therefore, the lack of pyridoxal phosphate, with its consequent decrease in kynureninase activity and accumulation of compounds usually removed by this enzyme, offers a qualitatively satisfactory explanation of the deranged tryptophan metabolism in pyridoxine-deficient animals.


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It is, however, necessary to show that, in pyridoxine deficiency, the rate of kynurenine formation exceeds that of kynurenine removal, before the observed reduction of kynureninase activity can be accepted as an adequate explanation for the excretion of kynurenine. The potential rates of kynurenine formation by the tryptophan peroxidase of rat liver have already been determined (Knox, 1951). The potential rates of kynurenine removal by kynureninase, in the livers of normal and pyridoxine-deficient rats, have now been determined for comparison. A new method of measuring the kynureninase activity was developed, and the properties of the enzyme were studied to ensure that this assay revealed the full potential activity of the enzyme. Measured under optimal conditions, the kynureninase activity of normal rat liver was higher than that previously determined (Braunshtein & Goryochenkova, 1949; Wiss & Hatz, 1949), and was, in fact, of a magnitude very similar to that of the tryptophan peroxidase. This similarity in the potential rates of kynurenine removal and formation under normal conditions is in keeping with the complete metabolism of tryptophan, without

accumulation of kynurenine, in normal rats. In livers of pyridoxine-deficient rats, the potential kynureninase activity was found to be decreased to about half, and was thus less than that of the tryptophan peroxidase. The ability to remove kynurenine in pyridoxine deficiency was therefore sufficiently depressed to account for the accumulation of some kynurenine and its derivatives by deficient animals on an unsupplemented diet. However, the very great accumulation of kynurenine and its derivatives, found when extra tryptophan is administered to pyridoxine-deficient animals, must be attributed largely to superimposition of the greatly increased kynurenine-forming potential, caused by the adaptive increase in the tryptophan peroxidase (Knox, 1951), upon the limited activity of the kynureninase.

## EXPERIMENTAL

*Preparation of enzyme.* Rat livers were disintegrated in 2 vol. of cold 0·14 M-KCl with a Waring Blendor and centrifuged in the cold. The supernatant (*S* 1) was acidified at 0° with 0·5 M-acetic acid to pH 4·8 (approx. 8 ml. of acid/100 ml. of *S* 1), promptly centrifuged in the cold and the supernatant (*S* 2) neutralized to pH 6·5. Cold ethanol was then added slowly to a final concentration of 45% (v/v) at −5°. The resulting precipitate (*A* 1) was collected by centrifugation at −5° and redissolved in half the original volume of ice-cold 0·14 M-KCl. The active fraction, which precipitated between 0·33 and 0·6 saturation with $(NH_4)_2SO_4$ (*AS* 2), was dissolved in a small volume of water and dialysed against running water at 5° for 12 hr. The protein content of each fraction was determined from the optical densities at 260 and 280 mμ. (Warburg & Christian, 1941). The procedure yielded about 50% of the initial activity in the final fraction (*AS* 2), and increased the activity/mg. of protein about 20-fold. The fractions retained their activities for several days at 2°.

*Assay.* An amount of kynureninase which would split not more than about 0·5 μmole of kynurenine/hr. was pre-incubated at 38° for 15 min. with 20 μg. of pyridoxal phosphate in a total volume of 1·0 ml. at a final concentration of 0·04 M-borate or phosphate buffer, pH 8·0. In some experiments, as noted, 10 μmoles of pyruvate or α-ketoglutarate were included in the reaction mixture to stimulate kynurenic acid formation. Experimental and blank tubes then received 0·2 ml. of $7·5 \times 10^{-3}$ M-L-kynurenine and 0·2 ml. water, respectively. One pair was immediately deproteinized by addition of 5 ml. of 1% (w/v) boric acid in ethanol, and a second pair after 40 min. incubation, for determination of the initial and final kynurenine concentration. Kynurenine was completely recovered by the boric acid-ethanol deproteinization, and its absorption spectrum in the neutral filtrate was not affected by slight changes in the pH of the reaction mixture. The filtrates and their appropriate blanks were read at 365 mμ. in a Beckman model DU spectrophotometer, and the kynurenine concentrations were calculated from the extinction coefficient of kynurenine in this medium ($\epsilon$ at 365 mμ. = 5150). The absorptions of kynurenic and anthranilic acids at 365 mμ. were less than 3% of that of kynurenine, and were neglected in the usual calculations of kynurenine concentration. The validity of this determination of kynurenine was confirmed by the results obtained with the diazotization procedure given below. The enzyme activity was expressed as the μmoles of kynurenine utilized/ml. of enzyme or mg. protein/hr.

*Paper-chromatographic identification of reaction products.* Alanine, kynurenic acid, anthranilic acid and residual kynurenine were identified in the reaction mixture by drying 0·5–1·0 ml. of the boric acid-ethanol filtrate on to a single spot on filter-paper sheets, which were then chromatographed in butanol-acetic acid-water according to the procedure of Dalgliesh (1952*a*).

*Determination of kynurenine and anthranilic acids by diazotization.* Diazotization of the reaction mixtures before and after ether extraction was used to confirm the spectrophotometric determinations of kynurenine and anthranilic acid. Anthranilic acid was quantitatively removed from 5% trichloroacetic acid filtrates by two extractions with 2 vol. of ether, and all the kynurenine was left in the aqueous phase. Extracted and unextracted filtrates and standard solutions of each compound were determined at the same time by the method of Bratton & Marshall (1939), with an extra 1 ml. N-HCl added to each tube. Diazotization was done at 0° and the colours were read exactly 90 min. later at 550 mμ. The colour formed in the extracted filtrate, and the extra colour found in the unextracted filtrate, were calculated from the appropriate standards as kynurenine and anthranilic acid, respectively.

*Spectrophotometric determination of kynurenic acid.* From the observed optical densities of the reaction filtrates at 365, 330 and 310 mμ., the percentage of kynurenic acid present could be calculated by the ratio method of Perry, Sutherland & Hadden (1950). This calculation, solved by the method of Crout (1941), also gave independent measures of the only other components absorbing in this region, namely kynurenine and anthranilic acid; the results agreed acceptably with the values determined by diazotization (Table 1). The adjusted matrix used in the calculations was constructed from the molar absorption coefficients: 5150, 2060, 894; 151, 8850, 3990; and 98, 2290, 3280; for kynurenine, kynurenic acid and anthranilic acid, at 365, 330 and 310 mμ., respectively. Only freshly dissolved solutions of kynurenine, free from the adventitious absorption developing with age in the region of 310 mμ., could be used as the substrate without introducing error into these determinations.

*Compounds.* $N^{\alpha}$-Acetyl-DL-kynurenine and DL-kynurenine (Dalgliesh, 1952*b*) were provided by Dr C. E. Dalgliesh, and αγ-diketo-γ-(*o*-nitrophenyl)-butyric and αγ-diketo-γ-phenylbutyric acids were provided by Dr A. Neuberger. The 3-hydroxy-DL-kynurenine (Butenandt, 1949) was a gift to Dr A. Neuberger from Prof. A. Butenandt, and the pyridoxal phosphate was a gift from Dr W. W. Umbreit. The disodium salt of αγ-diketovaleric acid was prepared by the method of Meister & Greenstein (1948). L-Kynurenine was isolated, after feeding L-tryptophan, from rabbit urine (Butenandt, Weidel, Weichert & Derjugin, 1943).

## RESULTS

*Reaction products of kynurenine.* Kynurenine disappeared primarily through the kynureninase reaction during anaerobic incubation with either crude or purified liver preparations. Only an-

Table 1. *The amounts of anthranilic and kynurenic acids formed from kynurenine by liver kynureninase preparations*

(Conditions as given in text. The products were determined by the spectrophotometric ratio method in all experiments, and in two of the experiments (the values in brackets) the kynurenine and anthranilic acid were also determined by diazotization. Pyruvate 10 $\mu$moles, where used.)

| Enzyme | (1) Kynurenine disappearance ($\mu$moles) | (2) Anthranilic acid formed ($\mu$moles) | (3) Kynurenic acid formed ($\mu$moles) | Kynurenine (1) accounted for as (2) + (3) (%) |
|---|---|---|---|---|
| Dialysed *S*1 | 0·62 | 0·48 | 0·11 | 95 |
| | (0·59) | (0·44) | (0·15)* | — |
| Dialysed *S*1 + pyruvate | 0·86 | 0·52 | 0·32 | 98 |
| *AS*2 | 0·86 | 0·76 | 0·10 | 100 |
| *AS*2 + pyruvate | 0·88 | 0·62 | 0·24 | 98 |
| | (0·83) | (0·58) | (0·25)* | — |

* Determination by difference from diazo values.

Table 2. *Relation of acylpyruvase and kynurenic acid-forming activities to the activity of liver kynureninase on purification*

(Fractions prepared and assayed as given in text. All activities were calculated as $\mu$moles of substrate/hr. Acylpyruvase assayed under (1) with $\alpha\gamma$-diketo-$\gamma$-(*o*-nitrophenyl)-butyrate; under (2) with $\alpha\gamma$-diketo-$\gamma$-phenylbutyrate. Purified acylpyruvase prepared by the method of Connors & Stotz (1949).)

| Fraction | Total kynureninase activities ($\mu$moles/ml./hr. × ml.) | Kynureninase purity ($\mu$moles/mg. protein/hr.) | Acylpyruvase/kynureninase (molar ratio of activities) (1) | (2) | Kynurenic acid formation (% of kynurenine disappeared) Without pyruvate | With pyruvate |
|---|---|---|---|---|---|---|
| *S*1 | 258 | 0·015 | 2·5 | 7·5 | 18 | 39 |
| *S*2 | 175 | 0·030 | 1·7 | 5·1 | 8 | 24 |
| *A*1 | 153 | 0·074 | 0·2 | 0·6 | 2 | 18 |
| *AS*2 | 126 | 0·303 | 0·05 | 0·15 | 9 | 28 |
| *AS*2 + acylpyruvase | 125 | — | 8·4 | 25·0 | 11 | 22 |

thranilic acid and alanine, the products of the kynureninase reaction, and a small amount of kynurenic acid were detectable chromatographically at the end of the reaction. The balance between the amount of kynurenine which reacted and the sum of the aromatic products formed, as determined by two different methods (Table 1), confirmed the essential absence of other reaction products. In the average preparation 80–90 % of the kynurenine which disappeared was converted to anthranilic acid by kynureninase, while the rest was accounted for as kynurenic acid.

The invariable formation of kynurenic acid raised slightly the apparent kynureninase activity as measured by kynurenine disappearance. The proportion reacting in this way was small and was fairly constant in different preparations, even after partial purification (Table 2), but could be doubled by the presence of pyruvate or α-ketoglutarate (Tables 1 and 2). The preliminary dialysis of crude preparations to be assayed for kynureninase activity minimized this reaction.

No evidence could be obtained that the kynurenic acid arose from the related $\alpha\gamma$-diketo acid, formed by a separate transamination of kynurenine or as a possible intermediate in the kynureninase reaction itself (Dalgliesh *et al.* 1951). Acylpyruvase, which could hydrolyse this hypothetical intermediate to anthranilic acid and pyruvate (Meister & Greenstein, 1948; Connors & Stotz, 1949), did not participate in the kynureninase reaction, nor when added in excess did it decrease kynurenic acid formation (Table 2). Acylpyruvase was assayed with $\alpha\gamma$-diketo-$\gamma$-phenylbutyrate and $\alpha\gamma$-diketo-$\gamma$-(*o*-nitrophenyl)-butyrate as substrates, in lieu of the unknown but similar derivative of kynurenine, $\alpha\gamma$-diketo-$\gamma$-(*o*-aminophenyl)-butyrate. The rates of hydrolysis of these two compounds by purified acylpyruvase (Connors & Stotz, 1949) were 15 and 5 %, respectively, of that of $\alpha\gamma$-diketovalerate. The ratio of acylpyruvase to kynureninase activities, both calculated as moles of substrate split per unit time, in the kynureninase preparations at various stages of purification, are given in Table 2. The initially higher activity of acylpyruvase was readily decreased by purification to well below that of kynureninase without affecting kynurenine disappearance or the formation of either anthranilic or kynurenic acids.

*Substrate concentration.* The effect of kynurenine concentration on the rate of its removal by a liver kynureninase preparation has been plotted in Fig. 1

according to the method of Lineweaver & Burk (1934). The enzyme was half-saturated ($K_s$) at $4 \times 10^{-4}$ M-L-kynurenine. It may be significant that the enzyme tryptophan peroxidase, which forms kynurenine in liver, had an identical $K_s$ (Knox & Mehler, 1950). The value for the analogous bacterial kynureninase was considerably lower ($3{\cdot}9 \times 10^{-5}$ M) (Hayaishi & Stanier, 1952). The substrate concentrations used in previous assays of animal kynureninase ($< 1 \times 10^{-3}$ M) were therefore initially suboptimal, and the progressive slowing down of the

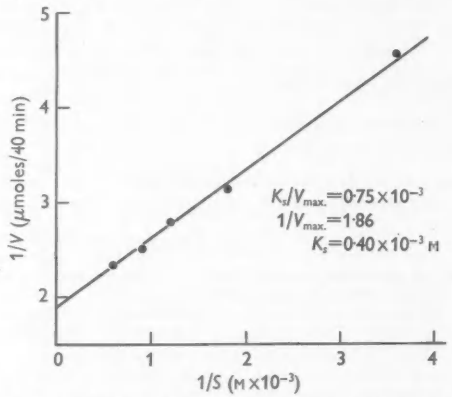


Fig. 1. The effect of kynurenine concentration ($S$) on the rate of the kynureninase reaction ($V$). 40 min. incubation of 0·5 ml. dialysed liver supernatant. ($V$ = μmoles kynurenine utilized/40 min.; $S$ = initial concentration of L-kynurenine, M × $10^{-3}$; $K_s$ = dissociation constant of enzyme-substrate complex.)

reaction, as the concentration fell during the long incubations, was mainly responsible for the low activities reported (Kotake & Nakayama, 1941; Wiss & Hatz, 1949), as was recognized by Braunshtein & Goryochenkova (1949). Halving the already inadequate L-kynurenine concentration, as when DL-kynurenine was substituted as substrate (Wiss, 1949), further reduces the kynureninase activity in the absence of any inhibition of the reaction by the D-component. Only L-kynurenine was used as substrate in these assays.

From the data in Fig. 1 it was calculated that the initial rate with $1{\cdot}25 \times 10^{-3}$ M-L-kynurenine, which was convenient to use, and the final rate after the reaction of 0·5 μmoles (i.e. with $0{\cdot}83 \times 10^{-3}$ M-kynurenine) would be 74 and 66% of the maximal kynureninase reaction velocity ($V_m$). The slowing of the reaction to this extent during the assay could not be detected by the difference measurements made, and these concentration limits were chosen for the assay of liver kynureninase. The maximal velocity with unlimited substrate could not be determined accurately by this method because of the intense absorption of kynurenine, but the $V_m$ was calculated to be about 1·4 times the rate actually measured. The values reported have not, however, been corrected by this factor.

pH *Optimum.* The maximal activity of rat liver kynureninase occurred at pH 8·0, which was nearer the optimum of bacterial kynureninase (pH 8·5) than the values previously reported (7·4 and 7·5) (Hayaishi & Stanier, 1952; Kotake & Nakayama, 1941; Braunshtein & Goryochenkova, 1949; Wiss, 1949). The rates at pH 6·5, 7·0, 7·4 and 8·5 were 25, 43, 81 and 77%, respectively, of the rate at pH 8·0. The spontaneous decomposition of kynurenine during incubation at pH values above 8·5 precluded measurements at more alkaline reactions. The reaction occurred equally well in phosphate and borate buffers, and no requirement for phosphate could be detected (Wiss, 1949).

*Coenzyme.* Even in an undialysed liver supernatant the kynureninase was apparently already partially resolved, since addition of pyridoxal phosphate increased the kynureninase activity by about 25%. However, dialysis alone did not increase the degree of resolution, so the activities found in homogenates after preliminary dialysis were assumed to be measures of the enzyme activity *in vivo*. One precipitation with ammonium sulphate followed by dialysis regularly produced about 70% resolution of the enzyme.

Maximal restoration of the activity of the partially resolved enzyme was obtained by pre-incubation with its coenzyme for 15 min. before the reaction. Thus a resolved enzyme preparation showed only 31% activity without pyridoxal phosphate, and 65% activity with pyridoxal phosphate added at the start of the reaction, compared with 100% activity when first pre-incubated for 15 min. with its coenzyme. Pyridoxal phosphate in 20 μg. amounts was in moderate excess over the requirement of all enzymes tested.

*Specificity.* Only the L-form of kynurenine was split by the enzyme. When 5·0 μmoles of L-kynurenine and of DL-kynurenine were separately allowed to react to completion with excess of enzyme, 4·53 and 4·83 μmoles of L-kynurenine and 2·46 and 2·47 μmoles of DL-kynurenine, respectively, had reacted after 60 and 100 min. When equal concentrations of L-kynurenine were tested, as L-kynurenine itself and as twice this concentration of DL-kynurenine to correct for the effect of substrate concentration upon the rate of reaction, no inhibition by the inactive D component was observed.

Only sufficient 3-hydroxy-DL-kynurenine was available to ascertain that it was split by kynureninase slightly more rapidly than was DL-kynurenine under the same conditions. The absorption spectrum of the products resembled that of a mixture of

3-hydroxyanthranilic and xanthurenic (4:8-dihydroxyquinolic-2-carboxylic) acids, but the products were not definitely identified. Both kynurenine and hydroxykynurenine accumulated in pyridoxine-deficient animals (Dalgliesh, 1952*a*), and since both reacted similarly with the enzyme, either or both could be the normal substrates of kynureninase. The reaction studied, therefore, represents the physiological mechanism by which both can be definitely metabolized through loss of their side-chains. The quantitative aspects of the reaction are probably similar with both compounds, but have in the present work been definitely established only for kynurenine.

The other side-chain-retaining metabolites identified as accumulating in pyridoxine-deficient animals (Dalgliesh, 1952*a*), kynurenic and xanthurenic acids and $N^a$-acetylkynurenine, were not substrates for kynureninase. $N^a$-Acetyl-DL-kynurenine was deacetylated, however, during incubation with the kynureninase preparations by a potent aliphatic deacetylase present (Bray, James, Raffan, Ryman & Thorpe, 1949), and the product could then be split by kynureninase. Purification of the kynureninase preparations did not eliminate this deacetylase, but its relative activity was decreased. The rate of hydrolysis of acetylkynurenine in the kynureninase reaction was consequently diminished as the deacetylase was decreased in the *S* 1, *A* 1 and *AS* 2 stages of purification to 44, 32 and 28 % of the rate of L-kynurenine hydrolysis.

*Assay of kynureninase.* From the properties of kynureninase described in the preceding sections, nearly optimal conditions for the enzyme assay were chosen, as given in the Methods. As calculated from the effect of the substrate concentration on the reaction rate, the reaction was substantially linear under these conditions until about 0·5 $\mu$moles of kynurenine had reacted. All assays were made within this limit, and the reaction was taken to be proportional to time and to the enzyme concentration (Fig. 2). Serial measurements of the disappearance of larger amounts of kynurenine during longer incubation periods showed, however, that the reaction was then first order.

The amount of kynurenine which reacted per unit time in this assay can be taken to represent the potential metabolic ability of the liver preparation examined to remove the original tryptophan side chain from the only compounds which can react in this way, kynurenine and hydroxykynurenine. The values obtained were subject to the qualifications that they were 10–20 % too high due to the formation of kynurenic acid being included, that they might be 1·4 times higher with unlimited substrate concentrations and that they might be slightly different if hydroxykynurenine were the more important substrate under physiological conditions.

Rat kidney, when tested for kynureninase activity by the same assay, was only about 10 % as active per unit weight as liver. Therefore both the formation of kynurenine and its removal occurred predominantly in the liver, and the rates of these reactions in liver will be of primary significance in determining the magnitude of tryptophan metabolism observed in whole animals.

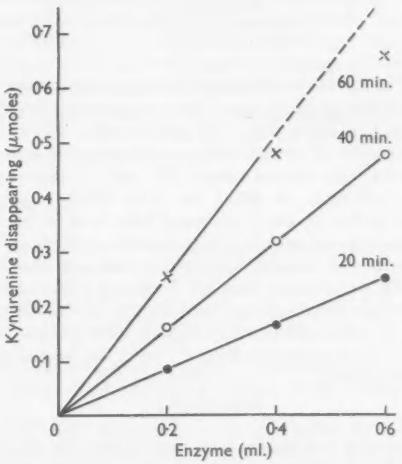


Fig. 2. The relation of kynurenine disappearance to enzyme concentration and to time of incubation. Dialysed supernatant fraction of liver (*S* 1) assayed as given under Methods for 20 min. (●); 40 min. (○); and 60 min. (×).

*Levels of kynureninase in rat livers.* Equal numbers of male and female rats were maintained for 3–6 months on the pyridoxine-deficient diet of Dalgliesh (1952*a*). Controls on the same diet with added pyridoxine were found to have the same enzyme activity as animals on a stock diet, and are not separately reported. No sex difference in the enzyme activity was observed. The assays were conducted in the system described, using 0·5 ml. of a freshly prepared 33 % (w/v) whole liver homogenate which had been dialysed for 2 hr. against running tap water. The activities/g. dry wt. of liver/hr., with and without added pyridoxal phosphate, found in the normal and the pyridoxine-deficient animals are given in Table 3. The kynureninase activity in the deficient livers was about half that of the normals, and since the livers of the deficient animals were smaller, their absolute kynureninase activity was much less than that of normal animals. With added pyridoxal phosphate the activity of the deficient preparations was restored nearly to normal, indicating that the enzyme protein was maintained during the deficiency.

Table 3. *Levels of liver kynureninase in normal and pyridoxine-deficient rats*

(Kynureninase determinations as described in text with and without addition of pyridoxal phosphate to liver homogenates. Activity expressed as μmoles kynurenine disappearing/hr./g. dry wt. Pyridoxal phosphate 20 μg., where used.)

| | Normal rats | | Pyridoxine-deficient rats | |
|---|---|---|---|---|
| | No addition | Pyridoxal phosphate added | No addition | Pyridoxal phosphate added |
| No. of animals | 10 | 40 | 7 | 5 |
| Average activity | 11·1 | 14·3 | 5·9 | 12·7 |
| S.E.M. | ±0·80 | ±0·03 | ±1·25 | ±0·71 |

The kynureninase activity was measured in the same way in the livers of two normal rats 6 hr. after they had been given intraperitoneally either 1 or 2 m-moles of DL-kynurenine. The activities found were in the normal range (12·6 and 15·7 μmoles/g. dry wt./hr.). A third rat 6 hr. after receiving 2 m-moles of DL-tryptophan also had a normal kynureninase activity. This treatment increased the activity of the tryptophan peroxidase in the liver from the normal value of 9·3 (Knox, 1951) to 30·5. The kynureninase could apparently not be adapted by its substrate, nor successively by tryptophan, as can the kynurenine-forming enzyme, tryptophan peroxidase.

## DISCUSSION

The rates of kynureninase activity in liver, in comparison with the previously determined rates of kynurenine formation, show that removal of the original tryptophan side chain by kynureninase can become the limiting step in tryptophan metabolism. When this occurs, it permits the accumulation of kynurenine and hydroxykynurenine, and their secondary conversion to the other metabolites containing this side chain which are excreted by pyridoxine-deficient animals. This interpretation rests upon the justifiable assumption that the full potential rates of enzymes measured *in vitro* bear some relation to the rates at which they can react *in vivo*. Under the condition of these experiments, the major reaction of kynurenine occurred in liver, where it is also formed, and consisted of conversion to anthranilic acid and alanine. The oxidation of kynurenine to hydroxykynurenine was not investigated, since either or both of these compounds may be the normal substrate of kynureninase, and the reactions of both are presumably very similar. The other tryptophan metabolites tested did not lose their side chain except secondarily by this reaction. In association with the kynureninase reaction, as measured, small amounts of kynurenic acid were formed. Its formation was largely dependent upon the presence of an α-keto acid such as pyruvate, but in the absence of pyruvate it was not a quantitatively important reaction and did not seriously affect the measurement of kynureninase activity. Only one enzyme was apparently involved in the kynureninase reaction itself and it was assayed under its optimal conditions, except that its maximal velocity with unlimited substrate concentrations was calculated to be about 1·4 times that actually measured. Subject to the qualifications mentioned, the kynureninase activity may be taken as a measure of the potential rate at which the side chain of tryptophan metabolites can be removed.

In the livers of normal and pyridoxine-deficient rats not supplemented with extra coenzyme the kynureninase activity was 11·1 and 5·9 μmoles/g. dry wt./hr., respectively. The rate at which kynurenine was formed from L-tryptophan in normal rat liver, and which was not changed in pyridoxine deficiency (Braunshtein & Goryochenkova, 1949), averaged 9·3 μmoles/g. dry wt./hr. (Knox, 1951). This comparison shows that kynurenine can potentially be formed and removed in normal livers at about the same rates. Halving the kynureninase activity, which occurred in the pyridoxine-deficient animals, might therefore result in the accumulation of a little kynurenine, and secondary conversion of it to other derivatives, to produce the small amounts of tryptophan metabolites excreted by pyridoxine-deficient animals not given supplementary tryptophan.

With the administration of extra tryptophan to animals, their tryptophan peroxidase activity can be raised at least tenfold by specific enzyme induction (adaptation), and under other conditions by hormonal action (Knox & Mehler, 1951; Knox, 1951), to increase greatly the kynurenine-forming potential. The kynureninase activity, however, was not found to be altered adaptively by administration of either kynurenine or tryptophan. The unchanged potential for removing kynurenine, together with the increased ability to produce it, can explain the excretion of kynurenine and its derivatives which occurs when even normal animals are given large doses of tryptophan (Porter, Stoerk & Silber, 1951). The still greater excretion of kynurenine and its derivatives seen when tryptophan is administered to pyridoxine-deficient animals must be attributed largely to this increased formation of kynurenine by the tryptophan-adapted

tryptophan peroxidase, superimposed upon the slower removal of kynurenine by kynureninase lacking an adequate concentration of its coenzyme.

## SUMMARY

1. Under optimal conditions, the average kynureninase activity of normal rat liver was 11·1 μmoles L-kynurenine removed/g. dry wt./hr. The activity in pyridoxine-deficient rats was decreased by lack of the coenzyme to 5·9 μmoles/g. dry wt./hr. The kynureninase was not increased adaptively in the animals by administration of kynurenine or tryptophan.

2. For comparison, kynurenine can be formed by the tryptophan peroxidase in normal rat liver at an average rate of 9·3 μmoles/g. dry wt./hr., and this rate can be adaptively increased tenfold by tryptophan administration to the animal.

3. The abnormal accumulation of tryptophan metabolites can therefore be attributed to a relative inadequacy of kynurenine removal, due to a decrease of kynureninase through lack of its coenzyme, or to an adaptive increase of the kynurenine-forming enzyme, tryptophan peroxidase, or to both. These mechanisms can account for the marked accumulation of metabolites in even normal animals given supplemental tryptophan, as well as in pyridoxine-deficient animals.

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# Studies on the Polarography of Proteins

## 1. THE RELATION OF WAVE HEIGHTS TO PROTEIN CONCENTRATION AND THE ORIGIN OF WAVE III

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When a protein is added to a polarographic cell containing a buffer, the polarogram consists of a single large wave which begins to rise at about −1·8 V. with reference to the saturated calomel electrode (S.C.E.). This wave was identified as a protein wave and its characteristics were described by Heyrovský & Babička (1930); it was called the prenatrium wave by Herles & Vančura (1932). Brdička (1933*a*) accidentally discovered a second protein effect. If the electrolyte contained ammonia and either bivalent or trivalent cobalt in addition to the protein, the reduction wave of bivalent cobalt was followed by two waves (frequently referred to as a double wave) which were due to the protein (Fig. 1). These last waves will be referred to as waves I and II respectively. He found also that, of a series of amino-acids tested, only cystine or cysteine produced a similar effect, for in an ammoniacal

cobaltous chloride solution each of these compounds gave rise to a wave which was in the same position as protein wave II. For a given molarity, cystine produced a wave that was twice the height of the corresponding cysteine wave (Brdička, 1933*b*). Brdička concluded that the disulphide cystine is reduced electrolytically to yield two cysteine molecules and that the protein current is due to the reduction of hydrogen ions which are supplied by the sulphydryl groups. The amino-acid or protein acts as a hydrogen-ion carrier.

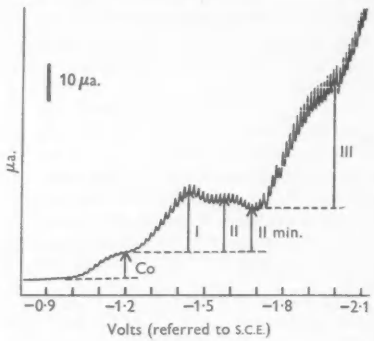


Fig. 1. A protein polarogram. Electrolyte contained bovine plasma albumin, 0·025 mg./ml., 0·5 M-$NH_4Cl$, 0·001 M-$CoCl_2$ and sufficient $NH_3$ to raise pH to 8·97. Arrows illustrate method of measurement of the components. Co is the reduction wave of $Co(H_2O)_6^{2+}$ and I, II and III indicate the three protein waves. $II_{min.}$ indicates the current at the minimum following wave II.

Some years later, Jurka (1939) pointed out that if the electrolysis was observed up to sufficiently high applied potentials, waves I and II were followed by a third wave (wave III) as shown in Fig. 1. By comparing the height of wave III with the height of the 'prenatrium' wave obtained in cobalt-free media, she showed that wave III of the complete polarogram in the presence of cobalt is the same as the 'prenatrium' wave.

The work described in the present series of papers arose from an independent discovery of wave III of insulin during an investigation of the effect of toxic metals on the polarographic protein waves. The author was not familiar with Jurka's paper at the time, and a series of experiments was initiated in order to establish that wave III was actually a protein wave and to study its characteristics. Brdička (1934) had shown previously that the relationship between the concentration of cystine and the height of the amino-acid wave followed a Langmuir adsorption curve. It was considered that if wave III gave a similar response then it was probably a protein wave also.

## TERMINOLOGY

All ions do not produce a polarogram of ideal shape. The record of most metallic ions is distorted by a 'maximum' which is usually a spike-like formation superimposed on the polarographic wave. It may have a rounded, hump-like shape. In practice, a maximum is undesirable since it interferes with the measurement of the wave height, but it can usually be eliminated by the addition of surface-active compounds to the solution. Thus the cobalt polarogram exhibits a maximum which is suppressed when protein is added to the cell. Depending upon the protein concentration and the pH and concentration of the buffer, the individual three protein waves may or may not show a humped appearance as though there were one or more maxima. It has always been assumed that the humps that appear on the protein waves are maxima in the sense in which this word is used in polarographic terminology. The author submits that we do not know enough about the cause of polarographic maxima or of the origin of the protein waves to accept this view without question and that it is advisable to speak of protein wave 'peaks' rather than 'maxima' until the question is settled. Well known maximum suppressors such as methyl red and other dyes, methylcellulose or gelatin, will eliminate the maxima that appear on the polarograms of metallic ions, but these substances fail to suppress the peaks or 'maxima' of the protein waves. The whole wave may be depressed but the peak remains.

Protein waves I and II are usually measured from the level of the cobalt limiting current up to the peaks of the waves. The magnitude of the current, measured from the cobalt limiting current, at the minimum which follows wave II may be the diffusion current of wave II; in one of his early papers, Brdička (1933*b*) used this terminology for the comparable point on the cystine wave. According to custom, the attainment of a diffusion current denotes a state in which the concentration of reactive ion has been decreased almost to zero in the layer of solution surrounding the drop, so that the current is limited by the rate of diffusion of reactive ion to the electrode and by the concentration gradient between the bulk of the solution and the electrode surface. It seems preferable to refer to the minimum that follows wave II as a minimum rather than a diffusion current. The protein current may be limited by the diffusion rates of cobalt and, under some circumstances, by the rate of diffusion of ammonium ion, but it is a matter of opinion as to whether the rate of diffusion of protein is of significance. Cobalt and probably ammonium ion are removed at the electrode surface but the protein is probably involved in an adsorption equilibrium at the electrode-solution interface.

## MATERIALS AND METHODS

Crystalline proteins commercially available were used for the study. Insulin was supplied by Dr Gordon Romans of the Connaught Medical Research Laboratories, Toronto. Pepsin, bovine plasma albumin, chymotrypsin and trypsin were obtained from Armour and Co., Chicago. Acetylated insulin was prepared by the method of Stern & White (1938) by passing keten into insulin suspensions (1 mg./ml.) in M-sodium acetate buffer (pH 5·7). The product was centrifuged, washed with acetone and ether, then dried in a desiccator over $H_2SO_4$. The proteins were dissolved in weak acid (about pH 3·5) and portions of this solution were added to the polarographic cell.

The borate buffer (pH 8) contained 0·05 M-$H_3BO_3$, 0·05 M-KCl and 0·004 N-NaOH.

The polarographic apparatus was assembled by the author and was of conventional design. A 'Speedomax' recorder (Leeds and Northrop Co., Philadelphia) was adapted for the measurement of current and a motor-driven Helipot potentiometer of 40 turns (Helipot Corp., S. Pasadena, Calif.) was used as a voltage divider. When the capillary was immersed in distilled water with a mercury pressure head of 50 cm., $m$ (flow of mercury) = 1·73 mg./sec., $t$ (droptime) = 4·07 sec. and the radius of the orifice was calculated as 23·7 $\mu$. according to Müller (1944). The electrolyte was maintained at a temperature of 25·0° ± 0·2° and $N_2$ gas was used to displace dissolved $O_2$. All pH determinations were made with a model G Beckman pH meter.

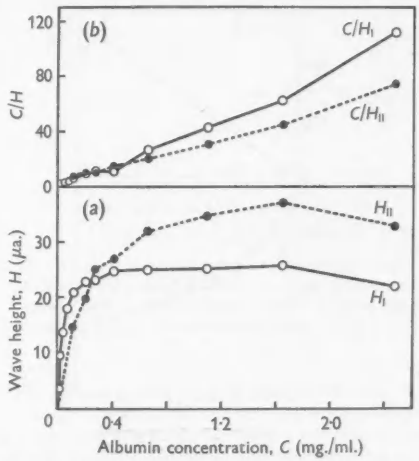


Fig. 2. (*a*) Relation between the heights of waves I and II ($H_I$ and $H_{II}$) and the concentration of bovine plasma albumin. The electrolyte contained 0·001 M-$CoCl_2$, 0·1 M-$NH_4Cl$ and sufficient $NH_3$ to give pH 9·6. (*b*) Relation between $C/H$ and $C$ to test for conformity to the Langmuir adsorption isotherm.

The method of measuring wave heights is illustrated in Fig. 1. In this paper and in those that follow, $H_I$ and $H_{II}$ refer to the magnitudes of waves I and II respectively, measured from the level of the cobalt limiting current up to the peaks of the individual waves. This is customary practice. $H_{II}$ min. refers to the magnitude of the current at the minimum which follows the peak of wave II. $H_{II}$ min. is also measured from the cobalt-limiting current. $H_{III}$ refers to the magnitude of wave III which is measured from the minimum following wave II to the peak of wave III.

## RESULTS

### *Relation between wave heights and protein concentration*

The height of each of the three waves for a series of protein concentrations has been determined with crystalline insulin, pepsin, bovine plasma albumin, chymotrypsin and trypsin. The protein concentration was altered by progressive dilution in the polarographic cell. The solution initially contained a relatively high concentration of protein (of the order of 2·5 mg./ml.), 0·001 M-cobaltous chloride, 0·1 M-ammonium chloride and sufficient ammonia to raise the pH to 9·6. The diluting solution lacked protein but had the same concentrations of cobalt and ammonia buffer as the initial solution. The values for the wave heights obtained with one protein, bovine plasma albumin, are reported in

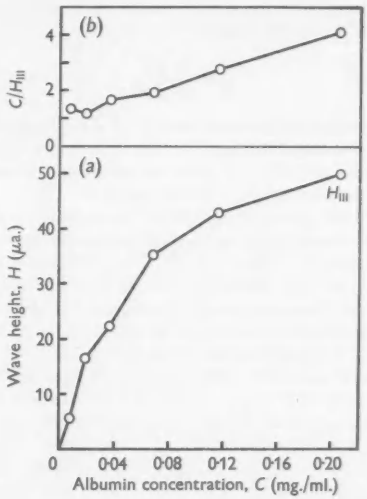


Fig. 3. (*a*) Relation between the height of wave III ($H_{III}$) and the concentration of bovine plasma albumin. The electrolyte contained 0·001 M-$CoCl_2$, 0·1 M-$NH_4Cl$ and sufficient $NH_3$ to give pH 9·6. (*b*) Relation between $C/H_{III}$ and $C$ to test for conformity to the Langmuir adsorption isotherm.

Figs. 2 (*a*) and 3 (*a*). It is evident that the magnitudes of waves I and II (Fig. 2) increased to a maximum value with increasing concentration of protein, but that these waves were depressed when the concentration exceeded a value of about 1·65 mg./ml. At protein concentrations greater than about 0·20 mg./ml., the shape of wave III made its measurement very difficult, so that the concentration scale in Fig. 3 is limited and the curve does not show a maximal value. Other proteins yielded curves similar in form but with individual differences with respect both to the rate of rise to the peak value and to the protein concentration at which the peak occurred. Under the conditions of these experiments wave I of all proteins attained a maximal value but wave II did so only in the case of bovine plasma albumin and chymotrypsin.

If the relationship between protein concentration and wave height resembles a Langmuir adsorption isotherm it should have the following general equation

$$H=\frac{aBC}{1+aC}, \qquad (1)$$

where $H$ is the wave height ($\mu$a.), $C$ is the concentration of protein (mg./ml.) and $a$ and $B$ are constants. A curve of this type approaches a limiting value $H_{max.}$ which is equal to $B$ when $C$ is large. The rate at which $H$ approaches $B$ is proportional to $a$. For practical purposes it is useful to rearrange the equation into the form

$$\frac{C}{H}=\frac{1}{aB}+\frac{C}{B}. \qquad (2)$$

If a straight line results from a plot of $C/H$ against $C$, then the data conform to the Langmuir type of equation and $B$ and $a$ can be calculated from the slope and intercept of the linear plot.

The concentration against wave-height curves obtained with the five proteins have been tested for conformity to equation (1). For illustration, the values of $C/H$ against $C$ for bovine albumin are given in the upper portions of Figs. 2 (*b*) and 3 (*b*). The points for waves I and II are reasonably linear up to a concentration of about 1·6 mg./ml. and wave III seems to follow equation 1 over the range in which this wave could be measured. Some proteins, such as insulin, give measurable waves at very low concentrations of protein and show a deviation from the Langmuir relationship in the low-protein range. Brdička (1933*b*) has observed this phenomenon with cystine and showed that it was due to adsorption of cystine on the walls of the electrolysis vessel.

The Langmuir constants $B$ and $a$ have been calculated for each curve. The constants vary considerably from one protein to another. Only in one instance did the actual wave height approach the theoretical maximum value, $B$. At the highest concentration of pepsin that was used (2 mg./ml.) the height of wave I was 22·2 $\mu$a. and $B$ for this curve was 22·3. None of the other curves attained the maximum value.

Depression of the wave height at high protein concentration was observed by Tropp, Jühling & Geiger (1939) and these investigators suggested that dehydration of the protein might be offered as an explanation. Another possibility is that the diminished wave is due to the adsorption of more than one layer of protein at the electrode-solution interface, the second layer attaching itself in such a way as to interfere with diffusion processes. That this is not a valid explanation is evident from the calculation of $B$ values. As the Langmuir equation has been applied, $B$ is equivalent to $H_{max.}$ which is proportional to the mass of protein adsorbed/unit area of electrode surface when the protein concentration is high and the surface is saturated. Since the observed wave heights approached $H_{max.}$ only in one case (pepsin wave I), there is no evidence of the formation of a second layer. The failure of other curves to approach $H_{max.}$ values is not to be construed as an indication that the surface was unsaturated at high protein concentration. The wave height would be depressed even though the surface were saturated if the high concentration of protein interfered with the rate of one or more electrode reactions. The following relationship supports this possibility.

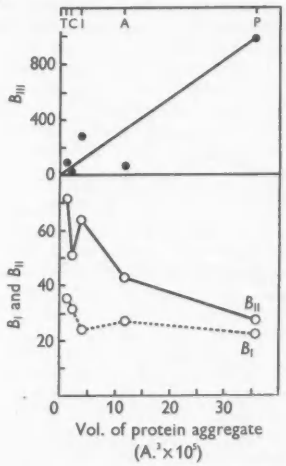


Fig. 4. The relationship between the volume of the protein aggregate and constant $B$ of the Langmuir adsorption isotherm. Subscripts I, II and III refer to waves I, II and III respectively. T, C, I, A and P refer to trypsin, chymotrypsin, insulin, bovine plasma albumin and pepsin respectively.

When the adsorbed molecules are large we might expect to find that the molecular size or the aggregate size would determine $B$ since the size would influence the number of molecules which could be crowded into a given area. In Fig. 4 the values obtained for $B_I$ and $B_{II}$ are reported and plotted against the volume of the protein aggregate as determined by X-ray diffraction (Fankuchen, 1945). The volume determined for horse serum albumin was used for the bovine plasma albumin studied. The volume of the trypsin aggregate was taken from Northrop, Kunitz & Herriott (1948).

$B_I$ and $B_{II}$ are small for proteins of large aggregate volume, as might be expected, since fewer large molecules can be accommodated on unit area of surface. However, comparison with the plot of

$B_{III}$ against aggregate volume (Fig. 4) suggests another interpretation. Wave III does not require the presence of cobalt or ammonia for its development and $B_{III}$ appears to become larger with increasing protein aggregate volume. The difference between the first two curves and that for $B_{III}$ may be due to the fact that large aggregates cover the electrode surface more efficiently and thus exclude cobalt-ammonia complexes from the electrode, or it may mean that the larger molecules interfere with the diffusion of complexes in the bulk of the solution.

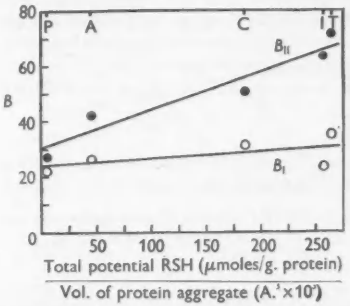


Fig. 5. The relation of the constant $B$ of waves I and II to the ratio: total potential RSH/protein aggregate volume. P, A, C, I and T refer to pepsin, bovine plasma albumin, chymotrypsin, insulin and trypsin respectively.

On the basis of work reported in a following paper (Millar, 1953) the author is convinced that other protein groups in addition to sulphydryl groups are involved in the production of the protein current, but that the sulphydryl groups probably have the greatest influence in determining the magnitudes of waves I and II and thus must be determinants of $B_I$ and $B_{II}$. In order to take this factor into account the various values of $B$ have been plotted in Fig. 5 against the ratio (total potential RSH/vol. of aggregate), where 'total potential RSH' is the sum of the reported cysteine and half-cystine residues in a given protein (Tristram, 1949). The term represents the total number of sulphydryl groups that would appear if all the cystine residues were reduced and is expressed as μmoles RSH/g. protein.

In Fig. 5, the straight lines are 'best' fits calculated by the method of least squares. $B_I$ and $B_{II}$ seem to be related to the ratio, but whereas the coefficient of correlation between $B_{II}$ and the ratio is 0·95 ($P = 0·02$), the coefficient between $B_I$ and the ratio is only 0·57 ($P = 0·5$) so that a relationship is not proved as far as $B_I$ is concerned. The relationship between $B_{III}$ and the ratio is not apparent. Indeed, the plotted points are widely scattered and suggest that groups other than sulphydryl groups are important determinants of the magnitude of wave III.

*The origin of wave III*

During the early stages of this investigation it was assumed, as a working hypothesis, that each of the three waves represented the release of hydrogen from three different protein side chains. If this assumption is valid, then it is reasonable to expect that the form of the polarogram could be altered if certain reactive protein groups were conjugated.

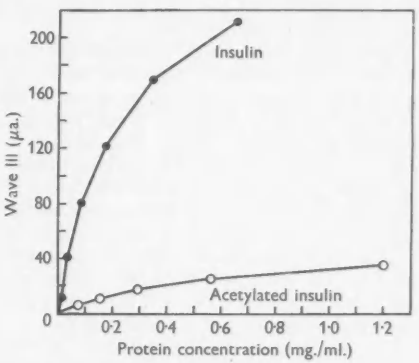


Fig. 6. The effect of acetylation of the amino groups of insulin on the height of wave III. The electrolyte contained insulin or acetylated insulin at the concentrations indicated, 0·001 M-$CoCl_2$ and ammonia buffer at pH 9·25 (0·1 M-$NH_4Cl$ and 0·1 M-$NH_3$).

Stern & White (1938) reported that insulin can be treated under mild conditions with keten gas in such a way as to acetylate free amino and phenolic hydroxyl groups. Amino groups are conjugated easily by treatment for 5 min. After 45 min. phenolic groups are affected, but they are not completely blocked until acetylation has proceeded for 30 hr. Polarograms of acetylated insulin prepared in this way were obtained for different concentrations of the protein. When the heights of the three waves were plotted against the protein concentration, the curves for acetylated insulin lay below those for insulin. The effect of acetylation on wave III is shown in Fig. 6. Acetylation of the protein for 5, 10 or 60 min. gave essentially the same curves and the data for these acetylated products were combined in order to calculate Langmuir equations to be compared with the equations for untreated insulin.

Exposure to keten diminished the value of the constant $B$ by 1·1, 4·5 and 80·3 % for waves I, II and III respectively, and the values of $a$ were diminished by 86·2, 62·2 and 71·9 %.

Since no wave was entirely abolished by acetylation another experiment was designed to determine whether the residual waves were due to hydrolytic removal of acetyl groups in the ammoniacal

electrolyte of the polarographic cell. Acetylated insulin at a concentration of 0·097 mg./ml. was allowed to stand in cobalt-ammonia buffer. Three polarograms of this solution were recorded 0, 160 and 385 min. after addition of the protein to the cell. During the period at alkaline pH the heights of waves I and II decreased 1 and 5·5 % respectively, while wave III increased 22·5 %. Thus, after 6 hr., wave III of the acetylated product was still only about one-quarter as large as the corresponding wave of insulin would have been.

These results indicate that free amino groups are involved to a considerable degree in the development of wave III, and confirmation of this evidence was sought in the repetition of an experiment described by Brdička (1936). Successive 0·1 ml. portions of 0·01 M-cystine in 0·1 N-sodium hydroxide were added to the polarographic cell, which contained 10 ml. of boric acid-sodium hydroxide buffer of pH 8·0. A polarogram was recorded after each addition. Since the system lacked both cobalt and ammonia, the catalytic sulphydryl wave which corresponds to protein wave II would not be anticipated. The polarogram of the buffer alone was flat until the discharge of sodium at about $-1{\cdot}9$ V., at which point the current rose sharply. The second record, obtained after the addition of 0·1 ml. of cystine solution to the cell (final cystine concentration $10^{-4}$ M) was flat until about $-1{\cdot}7$ V., then it too rose sharply. After five further additions of cystine, the current began to rise at about $-1{\cdot}6$ V. It was evident that $10^{-4}$ M-cystine caused the final discharge curve to shift about 0·2 V. to a more positive potential. Brdička obtained the same result and attributed the shift to the sulphydryl groups of cysteine which had been formed by the electrolytic reduction of cystine. Cystine did not produce a well defined 'prenatrium' wave, but Brdička interpreted the shift of the sodium current as being equivalent to a 'prenatrium' wave and concluded that the 'prenatrium' wave is another sulphydryl wave. However, there is no positive evidence to show that the shift was not caused by the amino or carboxyl groups of the amino-acid. Accordingly, the amino groups of cystine were blocked by preparing *NN'*-bis(2:4-dinitrophenyl)cystine according to the method of Abderhalden & Blumberg (1910). Since the disulphide of this conjugated product would be reduced to sulphydryl groups at the electrode, the latter groups could exert their effect in producing a shift of the sodium current but the action of the amino groups would be prevented. When the derivative was added to the boric acid-sodium hydroxide buffer a wave due to the nitro groups appeared with a half-wave potential of about $-0{\cdot}5$ V. At a concentration of $10^{-4}$ M, no shift of the sodium current could be detected. However, when the concentration was doubled a typical 'prenatrium' wave could be discerned which became more distinct and larger at higher concentrations. When ammonia gas was bubbled into the solution to pH 8·7 and cobaltous chloride added to a final concentration of $6{\cdot}8 \times 10^{-4}$ M, the 'prenatrium' wave was preceded by a large catalytic sulphydryl wave. Subsequent tests in which 1-fluoro-2:4-dinitrobenzene was added to the buffer explained these results. The reduction of the nitro groups on the benzene rings of the conjugate yielded amino groups (Astle & McConnell, 1943) and the 'prenatrium' wave was caused by the liberation of hydrogen from these newly formed groups.

A group of nitrogenous substances has been tested to determine whether this property is characteristic of these compounds. The results are summarized in Table 1.

Table 1. *The effect of some nitrogenous compounds on the potential at which sodium ion is discharged*

(0·1 ml. of 0·01 M solution of each compound was added to 10 ml. of borate buffer (0·05 M-$H_3BO_3$, 0·05 M-KCl, 0·004 N-NaOH; pH 8).)

| | |
|---|---|
| L-Lysine monohydrochloride | No effect |
| Methylamine hydrochloride | No effect |
| 1-Aminopropan-2-ol | No effect |
| Aniline | No effect |
| L-Histidine monohydrochloride | No effect |
| DL-Alanine | No effect |
| *m*-Aminoacetanilide | No effect |
| *p*-Bromoaniline* | No effect. 0·2 V. shift at pH 6·5 |
| L-Cystine† | 0·2 V. shift |
| Gelatin | 0·2 V. shift |
| 2:5-Diaminotoluene dihydrochloride | Catalytic wave |
| *NN'*-Bis(2:4-dinitrophenyl)cystine‡ | Catalytic wave |
| 1-Fluoro-2:4-dinitrobenzene | Catalytic wave |
| Pyridoxamine hydrochloride | Reduction wave at $-1{\cdot}45$ V. plus catalytic wave |

* Saturated solution (?) at final concentration.
† 0·0025 % (w/v) final concentration.
‡ $2 \times 10^{-4}$ M final concentration.

Under the conditions of the test, some had no influence, some shifted the sodium discharge to more positive potentials while others exhibited a true catalytic wave which resembled a protein 'prenatrium' wave. When a catalytic wave appeared, the pH of the electrolyte was altered by the addition of hydrochloric acid or sodium hydroxide. Enhancement of the wave by addition of acid and depression by alkali addition was used as a criterion of its catalytic nature. The electrolyte containing 2:5-diaminotoluene became brown so that the effect may be due to the oxidized form. Brdička stated that gelatin would produce no shift because it contains no cystine, but the author found that it did so when the gelatin concentration was 0·0025 % (w/v).

If wave III or the 'prenatrium' wave depends upon amino groups then the addition of formaldehyde to the protein-buffer system should depress the wave, since formaldehyde combines reversibly with amino groups. Formaldehyde itself is reduced at the dropping mercury electrode and the formaldehyde wave appears at voltages which interfere with the protein waves, so that the formaldehyde concentration must be minimal. The following experimental conditions were arranged to test the effect of formaldehyde. The 'prenatrium' wave of bovine plasma albumin (0·070 mg./ml.) was recorded in a solution of 0·1M-boric acid and 0·1M-sodium chloride.

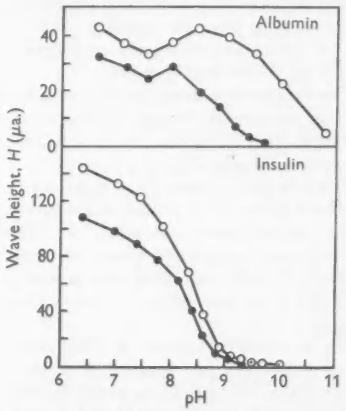


Fig. 7. The effect of formaldehyde on the 'prenatrium' waves of bovine plasma albumin and insulin at various values of electrolyte pH. ○—○, electrolyte contained initially 0·1M-$H_3BO_3$, 0·1M-NaCl and 0·070 mg. protein/ml.; ●—●, same plus $3·7 \times 10^{-4}$M-formaldehyde. pH was altered by adding 4N-NaOH to the initial electrolyte.

Successive additions of 4N-sodium hydroxide altered the pH and a recording was made after each addition. A second experiment was similar to the first except that the solution contained, in addition, formaldehyde at a concentration of $3·7 \times 10^{-4}$M. The results are shown in the upper portion of Fig. 7. In the presence of formaldehyde the 'prenatrium' wave is depressed over the entire pH range investigated. Below pH 8 it was evident that the wave was made up of more than one component and the shape of the polarogram made measurement of the wave difficult. For this reason it is impossible to determine whether the dip in the curves at pH 7·5 has any significance, although it occurred in both experiments.

Fig. 7 also shows the results of two experiments using insulin rather than plasma albumin. The insulin waves could be measured very easily. Again the wave was depressed in the presence of formaldehyde.

## DISCUSSION

Brdička (1947) has pointed out that conformity of the wave height-protein concentration curve to the Langmuir adsorption isotherm does not necessarily prove that the protein is adsorbed at the electrode surface since formal fulfilment of the equation might be the result of the rate of an electrode reaction which determines the height of the wave. This possibility must be acknowledged, but, until more is known of the electrode reactions involved in the catalytic protein waves, the implications of the Langmuir equation have been accepted at face value.

The typical polarographic protein response first shown by Brdička (1933*a*) is obtained only in the presence of cobalt and ammonia, and it is probable that it is the presence of the cobaltous hexammine ion, $Co(NH_3)_6^{2+}$, that is responsible for the effect. Brdička (1933*b*) has suggested that co-ordinate linkage between cobalt and the sulphur of sulphydryl groups allows the splitting of hydrogen from the sulphur so that it may be reduced at the electrode. It is probable, therefore, that proteins of large aggregate size depress the protein current by interfering with the free diffusion of cobalt-ammonia complexes.

It was gratifying to find a direct relationship between $B$ and total potential RSH/vol. of aggregate, and yet this result is surprising for it suggests that all the potential sulphydryl groups of the protein are available polarographically. The same conclusion is drawn from other data presented in a subsequent paper (Millar, 1953).

The three values of $a$ for acetylated insulin were small compared with those for the untreated protein. In the original development of the equation (Langmuir, 1918) $a$ is proportional to the fraction of molecules of adsorbate striking the surface which remain adsorbed, so we may assume that the free amino groups of the protein enhance the tendency of the protein to be adsorbed at the electrode surface. For this reason the wave height-protein concentration curves of waves I and II were depressed following acetylation, but the constants $B_I$ and $B_{II}$ were only slightly affected, and we may conclude that the influence of amino groups on waves I and II is indirect through their influence on the adsorption process. In contrast to these results, $B_{III}$ was depressed by 80% following acetylation and we may conclude that amino groups affect wave III through their influence on the adsorption process and also because they are involved in the electrode reactions which produce the wave III current. It is suggested, tentatively, that wave III depends on the following reactions:

$$RNH_3^+ + e \rightarrow RNH_2 + H, \qquad (1)$$

$$RNH_2 + H_3O^+ \rightleftarrows RNH_3^+ + HOH. \qquad (2)$$

These reactions are analogous to those proposed by Brdička (1933*b*) to explain the catalytic waves of cystine, cysteine and protein waves I and II. Reaction (1), the electrolytic reduction of hydrogen supplied by charged amino groups, provides the current of wave III. By reaction (2) the buffer regenerates charged amino groups which may participate once more in reaction (1). This interpretation is compatible with the known effect of altered buffer pH on this wave. Brdička (1936) found that the 'prenatrium' wave was enhanced by lowering the pH of the buffer.

The appearance of 'prenatrium' waves in the polarograms of many organic compounds confirms the interpretation presented above and shows that the 'prenatrium' wave is not a specific protein effect but that it is associated with nitrogenous groups. This hypothesis is supported by the work of Kirkpatrick (1947), although his data did not lead him to this particular conclusion. Kirkpatrick investigated the polarographic response of a series of alkaloids and he showed that many of these compounds produce catalytic waves which resemble protein 'prenatrium' waves.

Not all nitrogenous compounds yield 'prenatrium' waves and some do no more than shift the discharge curve of the alkali to more positive potentials, so that it is probable that the proximity of neighbouring groups and the presence of conjugated bonds are factors which determine the response. A point of particular interest, however, is that the presence of a sulphydryl group is not a prerequisite for the formation of a wave with the characteristics of a protein 'prenatrium' wave.

It will be recalled that acetylation of insulin did not completely eliminate wave III. The residual wave cannot be explained at present. Exposure of the acetylated product to the alkaline electrolyte for 6 hr. produced a slow enhancement of wave III, but the simultaneous gradual depression of waves I and II suggests that these changes were due to a small change in the pH of the electrolyte during the exposure period. There is no evidence that the residual wave III is due to the liberation of amino groups by hydrolysis unless acetate groups are removed very readily from a certain proportion of the amino groups during the recording of the first polarograms. It is possible that wave III depends on the presence of two different protein groups, one of which is blocked by short exposure to keten, perhaps an amino group, and another which cannot be blocked under these conditions and which is responsible for the residual wave of the acetylated product.

It is of interest to note that the insulin curves of Fig. 7 are shifted to the left with respect to the curves of plasma albumin. So far, it has not been possible to correlate the position of the curves with the amino-acid content of the proteins.

## SUMMARY

1. Polarograms have been recorded in 0·001 M-cobaltous chloride and ammonia buffer at pH 9·6 for the following proteins: insulin, pepsin, bovine plasma albumin, chymotrypsin and trypsin. The heights of all waves are reported.

2. The Langmuir adsorption isotherm equations have been calculated for the curves relating the wave height to the protein concentration for the three waves I, II and III.

3. The relation between the Langmuir constant $B$ and the volume of the protein aggregate suggests that the protein interferes with the diffusion of cobalt-ammonia complexes when the protein concentration is high and that the magnitudes of waves I and II are limited by the size of the protein aggregate.

4. $B_{II}$ is linearly related to the ratio (total potential sulphydryl groups/vol. of protein aggregate) which implies that all the potential sulphydryl groups of the protein are available polarographically.

5. Acetylation of insulin amino groups by ketene depresses the apparent adsorption of the protein on the electrode surface.

6. The depression of $B_{III}$ by acetylation of the protein or by the addition of formaldehyde to the electrolyte and the appearance of 'prenatrium' waves in the polarograms of certain organic compounds suggest that wave III depends upon the presence of nitrogenous groups rather than sulphydryl groups.

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# Studies on the Polarography of Proteins

## 2. THE INFLUENCE OF pH AND CONCENTRATIONS OF AMMONIA AND AMMONIUM ION UPON THE POLAROGRAPHIC WAVES


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If a protein is dissolved in a simple buffer, the polarogram of the system consists of a single large catalytic wave known as the 'prenatrium' wave (Heyrovský & Babička, 1930). Brdička (1933*a*) reported that if the protein was in an ammoniacal cobalt solution, the protein produced two waves which followed the cobalt reduction step. Later, Jurka (1939) found that the complete polarogram in ammoniacal cobalt solutions consisted of three waves which are referred to here as waves I, II and III respectively. Wave III has the same characteristics as the 'prenatrium' wave. It does not require the presence of cobalt.

Brdička (1933*a*) tested the effect of increasing the concentration of either the ammonia or ammonium chloride of the buffer in order to throw some light on the controlling factors of the system. When the concentration of either of these buffer components was increased, he observed enhanced protein waves. Cystine or cysteine in the same electrolyte produced a single wave at the same voltage as the second component (wave II) of the protein record, and he observed that alteration of the concentrations of the individual buffer constituents had an enhancing effect upon the amino-acid wave also (Brdička, 1933*b*). It is evident that the changes which Brdička observed had a complicated origin since his procedure altered the pH of the medium as well as the concentrations of the buffer components. The present author's interest in the effect of the concentration of the buffer components arose from a desire to test the effect of the electrolyte pH on the polarograms of proteins. It is obvious that the two questions are linked. Brdička (1936) studied the effect of altering the electrolyte pH upon the 'prenatrium' wave, but its influence upon waves I and II has not been reported.

### METHODS

The polarographic instrument and proteins used are described in the previous paper (Millar, 1953). Electrolyte pH values were measured with a Beckman Model G pH meter which was provided with a type 1190 E glass electrode for the major portion of the work. In the early experiments, portions of the solution were removed from the electrolytic cell, placed in the glass-electrode vessel for a pH determination, then returned to the cell. Later, a polarographic cell was constructed which would accommodate the pH-meter electrodes. These were connected to the pH meter by 30 in. shielded leads. The following procedures were used.

(*a*) The pH of an ammonia buffer depends upon the equilibrium $NH_4^+ \rightleftarrows NH_3 + H^+$ so that $[H^+]$ can be maintained at a relatively constant value and various concentrations of ammonium ion and ammonia used if $[NH_4^+]$ and $[NH_3]$ are maintained in constant proportion to one another by dilution of the buffer. The results reported in section A were obtained with this method. 0·001 M-$CoCl_2$ and 1·52 M-

$NH_4Cl$ were placed in the cell and $NH_3$ gas was bubbled into the solution until the pH was 8·7. Insulin was added to a final concentration of 0·070 mg./ml. A polarogram was recorded and the cell contents were then diluted with a solution which contained insulin and $CoCl_2$ at the above concentrations. A polarogram was recorded and further dilutions were made. In fact, the pH did not remain constant. It fell during the course of the experiment to a reading of 8·5, but in the subsequent calculations of the ammonia and ammonium ion concentrations, this discrepancy has been ignored. Subsequent experiments were arranged at three other pH values (9·0, 9·3 and 9·5). At each pH value, the original solution was diluted in eight or nine steps.

(*b*) The pH of the electrolyte was raised by adding small volumes of 18 N-NaOH to the cell containing protein (0·070 mg./ml.), $NH_4Cl$ (1·0 M) and $CoCl_2$ or Co $(NH_3)_6Cl_3$ (0·001 M). The results reported in section B were obtained with this method. As more and more alkali is added, the ammonium ion concentration falls and the ammonia concentration rises to the same extent. The sum of the concentrations of these components remains constant. Since $N_2$ gas must be passed through the solution after each addition of alkali to rid the solution of $O_2$ and for purposes of stirring, there was actually a small loss of $NH_3$, but for the present this discrepancy has not been taken into account.

(*c*) The pH of the buffer solutions was adjusted by varying the relative volumes of stock 0·4 M-$NH_4Cl$ and 0·4 M-$NH_3$ solutions. The final solution in the cell contained 0·001 M-$CoCl_2$ and insulin in varying amounts.

(*d*) The pH of the medium was altered by bubbling $NH_3$ gas into a solution containing $NH_4Cl$ (0·5 M), $CoCl_2$ (0·001 M) and bovine plasma albumin. The flow of gas was stopped at suitable intervals at which time the pH of the electrolyte was determined and a polarogram was recorded.

Preliminary experiments using procedures (*b*), (*c*) and (*d*) confirmed Brdička's observation that wave III is diminished as the pH is raised (Brdička, 1936). Waves I and II showed the reverse effect over the range of pH used.

## RESULTS

### A. *Experiments with varying concentration of total buffer components*

The relation between the heights of waves I ($H_I$) and II ($H_{II}$), and the calculated concentrations of ammonia and ammonium ion can be seen in Fig. 1 which shows that if the pH of the medium is held fairly constant, then both waves I and II become larger when the concentrations of ammonia and ammonium ion are increased. Fig. 1 also shows that at a given concentration of ammonium ion, waves I and II become larger when ammonia is added and the pH is raised. On the other hand, it is evident that, at a given concentration of ammonia, the heights of these waves are increased by additions of ammonium ion. Under these circumstances the pH falls. The waves seem to depend upon specific properties of ammonia and ammonium ion which mask any specific pH effect.

The curves relating $H_{II}$ and $[NH_3]$ are interesting, for, in contrast to the other families, they tend to be parallel and linear over an appreciable range.

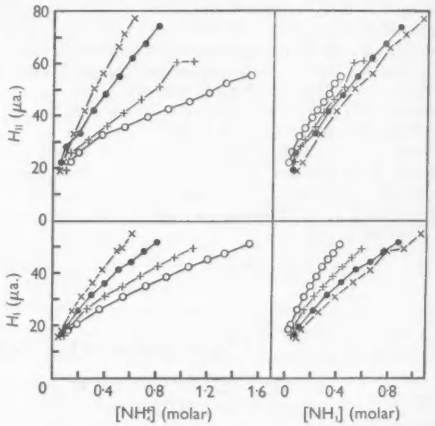


Fig. 1. The effect on waves I and II of alteration of the concentrations of ammonia and ammonium ion at relatively constant electrolyte pH values. Solutions contained bovine plasma albumin (0·070 mg./ml.), 0·001 M-$CoCl_2$, $NH_3$ and $NH_4Cl$. Initial buffer concentration was depressed by progressive dilution with a solution of albumin and $CoCl_2$ at the concentrations indicated above. pH values: ×—×, 9·5; ●—●, 9·3; +—+, 9·0; ○—○, 8·7.

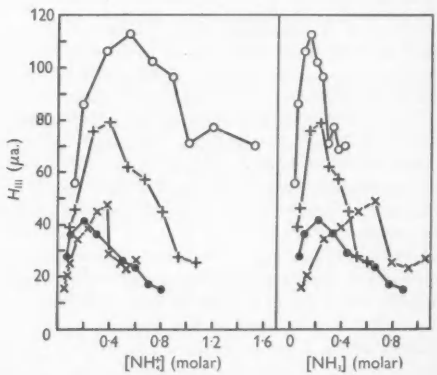


Fig. 2. The effect on wave III of alteration of the concentrations of ammonia and ammonium ion at relatively constant electrolyte pH values. Conditions and symbols as in Fig. 1.

Fig. 2 shows the relation between the magnitude of wave III ($H_{III}$) and the concentrations of the buffer constituents. This wave showed a distinctly different response from those of waves I and II. The

data are presented here in the interests of completeness, although the overall behaviour of this wave cannot be explained at the present time. As a confirmation of Brdička's work, however, it should be noted that at low molarity the height of the wave is enhanced by lowering the pH. At a given $[NH_4^+]$, a lower pH is produced by decreasing $[NH_3]$, while at constant $[NH_3]$, a lower pH is produced by raising $[NH_4^+]$. In the range of low buffer molarity at least, wave III does not seem to be influenced by any specific effects of the individual buffer constituents, and, of the three variables, pH seems to influence its height most. In contrast, any effect of pH on waves I and II is overshadowed by the effects of the individual buffer components.

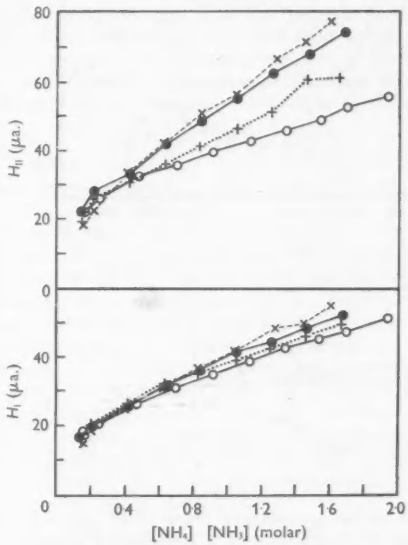


Fig. 3. The effect on waves I and II of ammonia buffer molarity at four values of electrolyte pH. Conditions and symbols as in Fig. 1.

Since an increased concentration of either ammonia or ammonium ion has an enhancing effect on the first two waves when the other component is maintained constant, the wave heights were plotted against the sum of the concentrations of these two constituents with the result shown in Fig. 3. This arrangement of the data shows that if the pH of the medium is altered by varying the relative concentrations of the buffer components while the total buffer molarity is maintained constant, then waves I and II are enhanced by increasing the pH of the electrolyte over the range which was used in this experiment.

### B. *Experiments with fixed concentration of total buffer components*

The results reported in section A suggested that the effect of pH on waves I and II could be investigated best under the conditions of procedure (*b*) in which the total buffer molarity remained constant.

Fig. 4 shows the results of an experiment where the initial solution contained M-ammonium chloride, 0·001 M-cobaltous chloride and crystalline chymotrypsin at a concentration of 0·070 mg./ml. Wave heights were measured for waves I, II and II min. The corresponding pH curves when the wave heights are plotted against pH are labelled $H_I$, $H_{II}$ and $H_{II\ min.}$ in accordance with the terminology used previously (Millar, 1953). To record a polarogram at pH 3·73, a small volume of concentrated hydrochloric acid was added to the solution. All other pH values were obtained by adding small volumes of 18 N-sodium hydroxide solution. All three curves have a positive slope as the pH of the medium is raised from 3·73 to about 8·6, after which they rise

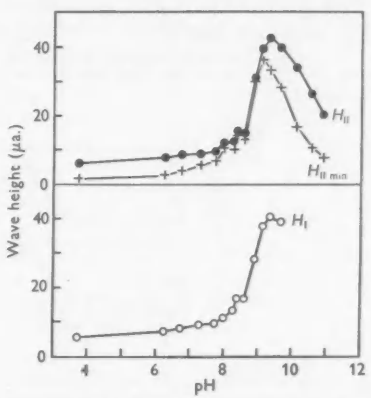


Fig. 4. Effect of electrolyte pH on the polarographic waves of chymotrypsin. For conditions see text.

rapidly to a maximum value, then fall. The curve for $H_I$ is incomplete because wave II was so large that wave I could not be distinguished properly. Similar pH curves were obtained with insulin, pepsin, and bovine plasma albumin. The pH curves of all the proteins showed the series of changes which are seen in Fig. 4, but the response of each protein differed from the response of the others in two respects. The magnitude of a given wave at a given pH value varied from protein to protein and the pH at which the peak of the 'hump' occurred was different for different proteins. For instance, the peak occurred at about pH 9·30 for $H_{II}$ of chymotrypsin, but it was at about pH 9·80, 10·25 and 9·85 respectively in the insulin, pepsin and plasma-albumin diagrams.

Fig. 5*a* shows the result of an experiment in which the cell contained M-ammonium chloride, 0·001M-cobaltic hexammine chloride and chymotrypsin at a concentration of 0·070 mg./ml. The pH of the cell contents was altered by adding small volumes of 18N-sodium hydroxide or concentrated hydrochloric acid. Thus the only difference between the conditions in this experiment and the one represented by Fig. 4 is in the valence of the cobalt in the two cases, but in contrast to Fig. 4, the three curves in Fig. 5*a* are quite flat in acid media. It was, in fact, a startling experience to record well developed protein waves in media as acid as pH 3·7. In electrolytes more acid than about pH 3·5, the true reduction wave of hydrogen ion distorts the protein record. In the trivalent cobalt medium it was possible to raise the pH to high values. This is not always feasible with bivalent cobalt for at a high pH there is an increasing tendency for the cobalt to precipitate.

Similar experiments were performed with crystalline chymotrypsinogen, insulin, pepsin and egg albumin. The results are shown in Fig. 5 (*b–e*). In each case the protein concentration was 0·070 mg./ml., and the solution contained M-ammonium chloride and 0·001M-cobaltic hexammine chloride. In no case was it possible to obtain a complete pH curve for wave I. At a high pH this wave becomes small and it is more or less merged with wave II.

## DISCUSSION

The reactions which are responsible for the catalytic waves of proteins, cystine, cysteine and certain other mercaptans are not well understood, but cobalt and ammonia are necessary constituents of the electrolyte if waves I and II are to be recorded and this leads to the supposition that a cobalt-ammonia complex must be an essential component of the system. The possibility that cobalt amalgam may lower the hydrogen overvoltage has not been excluded, but if this is the case it is difficult to identify the role of ammonia in the electrode processes unless it has a specific chemical effect on the protein or amino-acids or unless cobalt on the surface of the amalgam can form a complex with ammonia in the solution. The results are discussed, therefore, in relation to the electrode reactions which Brdička (1933*b*) has proposed to explain the catalytic waves.

Brdička (1933*a*, *b*) concluded that protein wave II is the result of the electrolytic reduction of hydrogen which is derived from sulphydryl groups

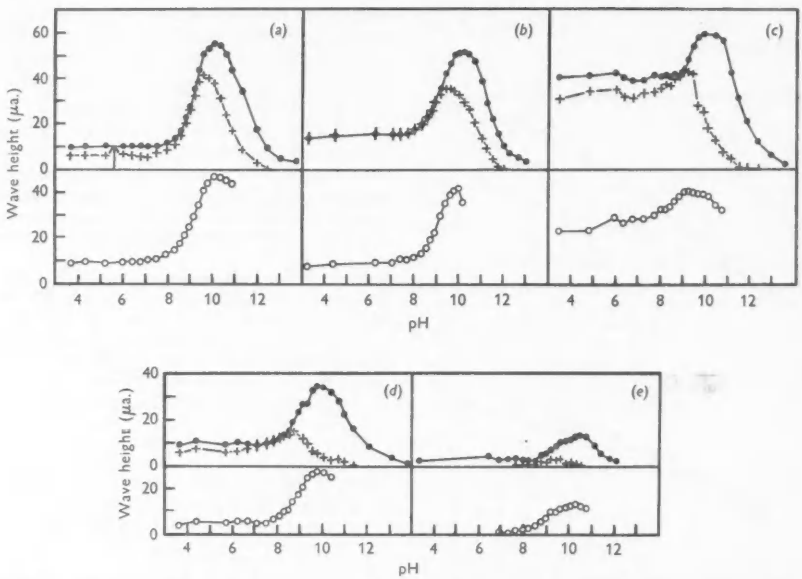


Fig. 5. Effect of electrolyte pH in the presence of trivalent Co on the polarographic waves of (*a*) chymotrypsin, (*b*) chymotrypsinogen, (*c*) insulin, (*d*) pepsin, and (*e*) egg albumin. For conditions see text. The method of measurement of $i_a$ is illustrated in (*a*). $H_I$, O—O; $H_{II}$, ●—●; $H_{II\ min.}$, +—+.

since cystine or cysteine in ammoniacal cobalt solutions produce a single wave at the position of protein wave II. He suggested that, as a result of co-ordination binding between cobalt and sulphydryl, the hydrogen can be removed readily from the sulphur and is reduced at the cathode according to reaction (1):

$$RSH + e \rightarrow RS^- + H. \quad (1)$$

He assumed that the sulphydryl anion reacts with water so that the sulphydryl group is regenerated leaving a hydroxyl ion which reacts with ammonium ion according to reactions (2) and (3):

$$RS^- + HOH \rightleftarrows RSH + OH^-, \quad (2)$$

$$NH_4^+ + OH^- \rightleftarrows NH_3 + H_2O, \quad (3)$$

RSH which is regenerated may then participate in reaction (1) once more. The sulphydryl groups may come from the free side chains of cysteine residues in the protein, or they may arise from the polarographic reduction of cystine residues at a lower applied potential according to the following reaction:

$$RSSR + 2H^+ + 2e \rightarrow 2RSH. \quad (4)$$

Brdička's pH studies (Brdička, 1936) are significant since the results constitute an important test of the current explanation of the origin of the polarographic protein waves, and yet these studies were applied to the 'prenatrium' wave rather than waves I and II, the waves which Brdička's three reactions were meant to explain. Furthermore, Brdička realized that an increase in the ammonia concentration should result in smaller waves I and II according to reaction (3), yet he observed the reverse effect and Fig. 1 confirms his observation. In the case of cystine he attributed this enhancement to the adsorption of increased quantities of amino-acid on the electrode surface (Brdička, 1933*b*). The following equilibria and reactions are pertinent to the discussion:

$$Co(NH_3)_6^{3+} \rightleftarrows Co^{3+} + 6NH_3, \quad (5)$$

$$Co(NH_3)_6^{3+} + e \rightarrow Co(NH_3)_6^{2+} \text{ (first cobalt wave)}, \quad (6)$$

$$Co(NH_3)_6^{2+} + 6H_2O \overset{\text{rapid}}{\rightleftarrows} Co(H_2O)_6^{2+} + 6NH_3, \quad (7)$$

$$Co(H_2O)_6^{2+} \overset{\text{slow}}{\rightleftarrows} Co^{2+} + 6H_2O, \quad (8)$$

$$Co(H_2O)_6^{2+} + 2e \rightarrow Co + 6H_2O \text{ (second cobalt wave)}, \quad (9)$$

$$RSH + e \xrightarrow{Co(NH_3)_6^{3+}} RS^- + H \text{ (protein wave)}, \quad (10)$$

$$RS^- + H^+ \rightleftarrows RSH, \quad (11)$$

$$NH_4^+ \rightleftarrows NH_3 + H^+. \quad (12)$$

Protein waves I and II can be obtained in an ammonia buffer if the electrolytic cell contains either cobaltous chloride or cobaltic hexammine chloride. Reaction (5) represents the dissociation equilibrium of the cobaltic hexammine ion. This is a very stable complex with a dissociation constant of $2{\cdot}2 \times 10^{-34}$ (moles/l.)[6] according to Lamb & Larson (1920). In the polarographic cell this ion produces a wave at about $-0{\cdot}4$ V. referred to the saturated calomel electrode (S.C.E.) (Willis, Friend & Mellor, 1945). The electrode reaction is represented in reaction (6). Such a solution will contain no cobaltous hexammine ion except that which is 'deposited' at the electrode by the reduction of the trivalent ion. The bivalent complex is distinguished from the trivalent complex by its instability, for Lamb & Larson (1920) concluded that its dissociation constant is of the order of $1{\cdot}25 \times 10^{-5}$ (moles/l.)[6]. This means that its dissociation curve lies almost entirely between pH 8 and 9. The dissociation is represented in reaction (7). Reaction (7) moves to the left when cobaltous chloride is added to an ammonia buffer so that it is apparent that the pH of the medium will determine the concentration of cobaltous hexammine ion in the bulk of the solution in this case. The hydrated cobalt ion which is in close proximity to the mercury drop is reduced according to reaction (9) (Willis *et al.* 1945), to produce the second cobalt wave. The consequent lowering of the concentration of hydrated ion will draw reaction (7) to the right and the bivalent ammonia complex will dissociate in the neighbourhood of the drop. The dissociation is apparently rapid since the second cobalt wave is twice the height of the first cobalt wave. Brdička (1933*a*) stated that the second cobalt wave is preceded by a very small wave which he attributed to the reduction of free bivalent cobalt ions which are represented in reaction (8). Because the wave is small, the dissociation of the hydrated complex must be slow. Reactions (10), (11) and (12) represent a slight revision of the reactions which Brdička (1933*b*) proposed to explain the catalytic protein waves I and II. Co-ordinate bonding between the cobalt of the cobaltous hexammine ion and mercaptan sulphur allows the hydrogen to be removed and reduced (reaction 10). The sulphydryl group is restored through reaction (11) and the group may then enter into reaction (10) once more. The objection may be put forward that reaction (11) does not differ from the combined effect of reactions (2) and (3) and this is true, since in either case the hydrogen ion must be supplied from the water through the action of the buffer. The advantage in reactions (10) and (11) lies in the fact that they place emphasis on the role of ammonia in the maintenance of a supply of cobalt-ammonia complex. In parenthesis it should be noted that we still have no explanation for the observation of Brdička (1933*a*, *b*), that whereas protein polarograms can be recorded from ammoniacal solutions containing

either bivalent or trivalent cobalt, cystine and cysteine catalytic waves can be produced only from ammoniacal solutions containing bivalent cobalt.

Reaction (12) represents the ammonia buffer equilibrium. It has a pK of 9·26.

If we assume that reaction (5) is essentially independent of pH, then the hydrogen-ion concentration of the buffer can affect this scheme only at reactions (7) and (11). When the pH is raised the protein current will tend to be depressed since the regeneration of sulphydryl groups (reaction 11) will be hindered. On the other hand, the resultant increase in ammonia concentration will tend to force reaction (7) to the left so that there will be a greater probability of sulphydryl activation by cobaltous hexammine ion. It seemed conceivable that if protein polarograms were recorded over a wide range of pH the wave heights might be limited by reaction (7) in media with pH values below 9, and by reaction (11) in media of some higher pH value. Cannan (1942) has suggested that the sulphydryl groups in proteins may have a pK of about 10.

In the electrolyte which contained bivalent cobalt the pH curves always showed a definite positive slope when the pH was low, and this behaviour is accepted as evidence that the wave height is limited by reaction (7) in this pH range. In such a system reactions (5) and (6) are not present, and the concentration of cobaltous hexammine ion depends on the pH of the medium through reactions (7) and (12). At low pH the rate of supply of this complex to the electrode will be low because there will be only a fraction of the total cobalt in this form. As the pH is raised and a greater proportion of the cobalt assumes the hexammine form larger waves would be anticipated. It was expected that this portion of the curve might become flat if cobaltic hexammine chloride was used, because the reduction of the trivalent complex ion would supply bivalent complex ions to the electrode surface at a constant rate which would be determined by the initial concentration of trivalent ion and its diffusion constant. This expectation was realized in the experiments represented by Fig. 5. Presumably most of the bivalent complex dissociates (reaction 7) to provide hydrated complex which is reduced according to reaction (9), while some of the bivalent complex co-ordinates with mercaptan to provide activation for reaction (10).

According to this viewpoint it might be expected that the wave height would drop close to zero below pH 8 in a bivalent cobalt solution since the dissociation curve of cobaltous hexammine ion lies almost completely between pH 8 and pH 9. However, it must be realized that the pH determinations refer to the pH of the bulk of the solution, whereas the wave heights depend upon the pH of a thin layer of solution which surrounds the cathode. This thin layer of solution may be referred to as the reaction layer. When the bulk of the electrolyte has a pH value which lies within the functional range of the ammonia buffer (pH 7·25–11·25 approximately), the pH of the reaction layer is probably close to the Beckman pH-meter reading, but beyond the effective range of buffer the pH of the reaction layer is probably always higher than the Beckman reading. This is because the electrode process removes hydrogen ions from this volume and at the same time six ammonia molecules are set free at the electrode surface for every cobalt ion reduced. The pH of the reaction layer does not rise indefinitely, however, since the components of a buffer are present. Ammonia probably diffuses away from the drop and ammonium ion probably diffuses to it until an equilibrium has been attained. The final pH of the reaction layer will depend on the diffusion constants of these buffer components, on their concentrations in the bulk of the electrolyte, on the rate of liberation of ammonia from cobalt complexes and on the protein current itself, which removes hydrogen ions. It would be expected to vary from wave to wave. The fact that the wave heights do not fall to zero in electrolytes having pH less than 8 simply means that the alkaline conditions in the reaction layer force reaction (7) to the left and provide some of the bivalent complex in this region even though there may be very little in the bulk of the solution. When the cobaltic hexammine salt is used it seems likely that when the pH of the bulk is neutral or acid, the pH of the reaction layer is indicated by the pH at which the curves cease to be linear. For instance, in Fig. 5*a* the pH of the reaction layer was about 6·75 when wave I was recorded from media with pH values more acid than this. Similarly, the pH of the reaction layer was about 7·5 when $H_{II}$ and $H_{II\ min.}$ were recorded, although the bulk of the cell contents was much more acid.

The pH curve of wave I of insulin is not flat in neutral or acid media (Fig. 5*c*). This deviation from the usual response cannot be explained, but it may be related in some manner to the high cystine content of insulin or to the dissociation of insulin which is said to occur when the pH is raised (Gutfreund, 1948; Millar, 1953).

The same general pattern is observed in all these charts, yet each shows individual differences which must be related to the physical and chemical characteristics of the individual proteins. The most obvious differences are in the magnitude of the current throughout the flat portions of the curves. This measurement will be referred to by the symbol $i_a$. Another obvious difference between charts is the position of the peak of the 'hump' on the pH scale and, lastly, the magnitude of the current at the peak of the 'hump'.

An attempt was made to learn something of the factors which determine the shape of the pH curves by plotting these characteristics of the pH curves against the amino-acid content of the proteins. In certain cases a correlation has been found. Excessive weight cannot be placed upon these correlations, because some of the data are no more than approximate. Nevertheless, all these plots point in one direction and they are, therefore, suggestive.

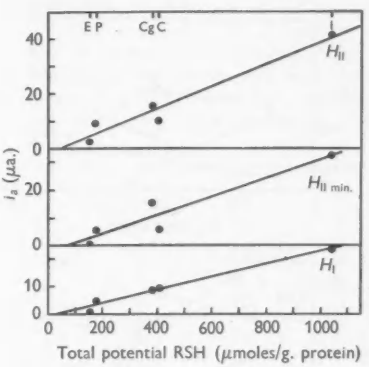


Fig. 6. The relation between $i_a$ and the total potential sulphydryl content of five proteins. $i_a$ is the wave height obtained in electrolytes of relatively acid pH as shown in Fig. 5. E, P, Cg, C and I refer to egg albumin, pepsin, chymotrypsinogen, chymotrypsin and insulin respectively.

In Fig. 6, the various values for $i_a$ have been plotted against the 'total potential RSH' of each of the proteins as defined in the first paper of this series (Millar, 1953). This aggregate figure is used because the polarograph does not distinguish between cysteine sulphydryl groups and those groups which arise from the reduction of cystine. The straight lines are best fits calculated by the method of least squares.

Fig. 6 shows that there is a strong correlation between $i_a$, the height of the level portion of the pH curve, and the potential sulphydryl content of these proteins. This is particularly true of the $i_a$ of $H_I$ and yet certain difficulties arise when we try to explain this linearity. Such a relationship is possible only because, in each experiment, the same concentration of protein was used in each case, namely, 0·070 mg./ml. This condition, in fact, converts the abscissa into a sulphydryl concentration scale if we look upon the protein simply as a carrier of mercaptan. For instance, the total potential RSH content of insulin is 1·04 m-moles/g. of protein (Tristram, 1949) so the concentration of potential RSH in the solution was 0·0728 μmoles/ml. solution. If this viewpoint is valid then we conclude that $i_a$ is linearly related to the concentration of potential RSH in the solution. The diffusion current of metal ions and many organic compounds is usually directly proportional to the concentration of reactive ion also, but since the RSH of the protein is probably not eliminated at the electrode, but is being continually regenerated there, it is doubtful if the rate of diffusion of the protein 'carrier' limits the current. Nor can the linearity be explained by assuming that the quantity of protein adsorbed on the electrode is proportional to the potential RSH content. This would lead to a parabolic relationship. The conclusion that seems most likely is that approximately the same quantity of protein is adsorbed on unit area of the electrode no matter which protein is being used and that either all of the potential RSH groups in each protein react at the drop or that a certain fixed proportion, say 50 %, of the groups of each protein is involved in the reaction. Under a given set of experimental conditions with respect to buffer and cobalt concentration and at low electrolyte pH, the current would be limited by the rate of diffusion of hydrogen ions except for the fact that the electrode process causes the pH of the reaction layer to rise until the buffering properties of the electrolyte are encountered. The greater the number of potential RSH groups available, the greater is the rate of discharge of hydrogen ions and the more closely does the pH of the reaction layer approach the pK of the buffer. Thus adequate quantities of hydrogen ions are supplied to support the electrode reaction.

Certain objections can be raised against this proposal which cannot be answered yet. Denaturation of a protein usually enhances its polarographic waves (Wenig & Jírovec, 1938). This phenomenon is usually explained as being due to a 'release' of previously unreactive sulphydryl groups. This explanation is not valid if all the potential sulphydryl groups are active in the untreated protein, and there is certainly no evidence to support the view that a certain fixed proportion of the potential groups of different proteins are more reactive than the remainder. However, it must be emphasized that the polarograms we are concerned with here were recorded under special conditions while all the denaturation studies involved electrolysis at conventional values of electrolyte pH fixed in the neighbourhood of 9·3. If it were found that denaturation of a protein shifted its pH curve toward more acid values, enhancement of the waves by denaturation could be explained without postulating the existence of polarographically inactive sulphydryl groups in the untreated protein.

It is probable that the protein adsorbed on the drop is denatured to some degree because of the spreading and the alkaline environment. It may be that this denaturation contributes to the linearity of the data.

Fig. 6 implies that the 'turnover number', the number of reactions in which a single sulphydryl group participates in 1 sec., is a constant for different proteins. This seems reasonable, but there has been no previous evidence on this question one way or the other.

The results portrayed in Fig. 6 invite further investigation. If a linear relationship holds for a wide range of protein concentrations and for mixtures of different proteins, it may be of some analytical value, for a very simple procedure would be required. Since trivalent cobalt is used, an ammonia buffer is not essential and there might be some advantages is using other buffers. If further

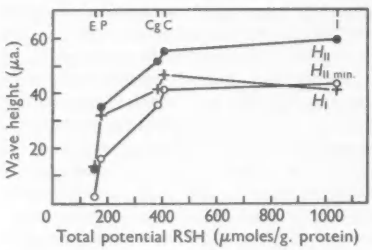


Fig. 7. The relation between the magnitude of the wave height at optimal pH (see Fig. 5) and the total potential sulphydryl content of five proteins. E, P, Cg, C and I refer to egg albumin, pepsin, chymotrypsinogen, chymotrypsin and insulin respectively.

studies confirm this result it will be the first time that the height of any of the protein waves has been correlated quantitatively with the chemical composition of the protein. Further attempts to correlate $i_a$ with the amino-acid contents of these proteins were not successful.

In Fig. 7, the magnitude of the current at the peak of the 'hump' is plotted against the total potential RSH of each protein. These curves bear a resemblance to the wave height-protein concentration curves that are obtained in the usual ammonia buffer at constant electrolyte pH in the neighbourhood of pH 9·2–9·5 (Millar, 1953). A comparison of Figs. 6 and 7 suggests (*a*) that the electrode reactions are different within these two pH ranges, or (*b*) that the large current at high pH causes some component of the system to be depleted so that it limits the current.

In Fig. 8, the pH at which the peak of the 'hump' occurs is plotted against the aggregate arginine and lysine content of the proteins (Tristram, 1949). Analytical values for the lysine content of chymotrypsin could not be found, so only four proteins are represented. There seems to be a fair correlation between these two variables except in the case of wave I. The same statement may be made when the pH of the peak is plotted against the number of free amino groups/mole of protein, although even less weight can be given to this observation since there are analytical data available for only three of the five proteins. There is some degree of correlation between the pH of the peak and the tyrosine content of the proteins (Tristram, 1949). It seems to be best in the case of wave I (Fig. 8).

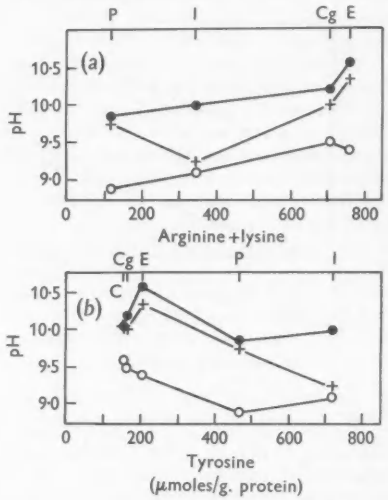


Fig. 8. The relationship between the optimal electrolyte pH and the content of (*a*) arginine + lysine, (*b*) tyrosine residues in some proteins. P, I, Cg, C and E refer to pepsin, insulin, chymotrypsinogen, chymotrypsin and egg albumin respectively. $H_1$, +—+; $H_{II}$, ●—●; $H_{II\ min.}$, ○—○.

It is not possible to determine with a high degree of accuracy the pH at which the pH curves attain their maximum value, but it is probably safe to conclude that the higher the proportion of basic groups and the lower the proportion of tyrosine groups in a given protein, the greater is the likelihood that the pH curve of that protein will have a maximum value at a high pH. It is interesting to note in this connexion that the tyrosine side chain, in its ionized form, has a negative charge, while the ionized forms of the side chains of arginine and lysine have a positive charge.

A second objective of this work was to determine whether the current would be limited by the rate of regeneration of sulphydryl groups (reaction 11) when the pH of the electrolyte is high. If, at high pH, the current fell from the $i_a$ level along a conventional dissociation curve, there would be some justification for saying that the current was limited by reaction (11) since a correlation had been found

between $i_a$ and the total potential RSH content of the protein. The question is made more complicated by the appearance of the 'hump' on the pH curve. It is not surprising to find that the heights of the waves are enhanced when the pH of the electrolyte is in the neighbourhood of the pK of the buffer system, for in such circumstances the electrolyte can supply hydrogen ions at a maximal rate for the regeneration of sulphydryl groups. However, it is surprising to find that the peaks of the 'humps' are all on the alkaline side of the pK of the buffer (pK = 9·26). To show that this discrepancy was not due to instrumental errors, the individual volumes of 18N-sodium hydroxide which had been added to the cell were divided by the increment of pH which

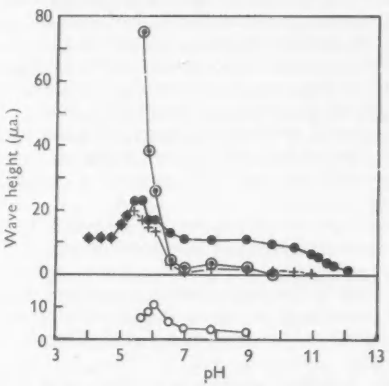


Fig. 9. Effect of electrolyte pH on the polarographic waves of insulin with sodium acetate buffer. For conditions see text. $H_I$, O—O; $H_{II}$, ●—●; $H_{II\ min.}$, +—+; $H_{III}$, ⊙—⊙.

each had produced and the quotients were plotted against a pH scale. The resultant curves were not smooth because of the strength of the base and the small volumes that were added, but it was clear that all five curves had maximum values that were close to pH 9·26. These curves, which are a measure of the buffering power of the system, are the $\beta$ curves of Van Slyke (1922).

If the 'hump' appeared on the acid side of the buffer pK the phenomenon might be explained as follows. Since the reaction layer is more alkaline than the bulk of the solution, the peak of the buffer $\beta$ curve was attained in the reaction layer when the pH of the bulk of the solution was below the pK value. Since the current rose on the acid side of electrolyte buffer pK, the current must depend upon the $\beta$ of the buffer. However, the hump actually occurs on the alkaline side of the buffer pK. The above argument might therefore be used to show that the current is not directly dependent on the buffer $\beta$, but this does not constitute an explanation for the position of the 'hump'.

The 'hump' seems to be superimposed on the sulphydryl dissociation curve which would be anticipated by our hypothesis in its simplest form. The 'hump' is not a simple buffer $\beta$ curve, and yet the buffering power of the system is involved in its formation as the following experiment demonstrates (Fig. 9). Here the cell contained sodium acetate (0·1M), cobaltic hexammine chloride (0·001M) and insulin (0·070 mg./ml.). The solution was made alkaline to pH 12·1 by the addition of an appropriate amount of 18N-sodium hydroxide, then

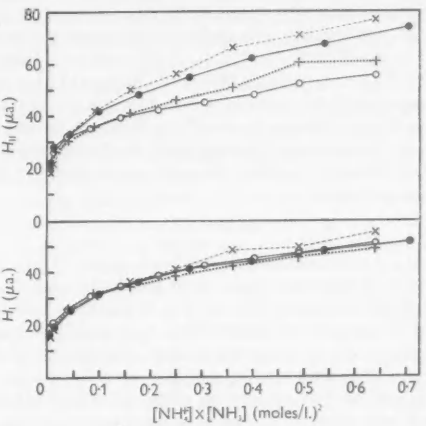


Fig. 10. The relation of the product of the concentrations of the buffer components to the heights of waves I and II of bovine albumin for four values of electrolyte pH. Conditions and symbols as in Fig. 1.

polarograms were recorded at various stages as the pH was lowered by additions of concentrated hydrochloric acid. (Fig. 9 includes the response of protein wave III as well as the other three pH curves.) The pK of the acetate buffer is 4·75 and the 'hump' of each pH curve occurred on the alkaline side of the buffer pK. At higher pH, $H_{II\ min.}$ fell to zero, but $H_{II}$ maintained its height up to about pH 9·0, although the bulk of the electrolyte was not buffered at pH values higher than about 6·75. However, the reaction layer surrounding the electrode was probably buffered to some extent by an ammonia buffer, since ammonia was liberated there by the reduction of the trivalent cobalt complex. At pH values higher than 9·0 or 9·25, $H_{II}$ fell toward zero. These curves show that a 'hump' is associated with the buffer system and that it does not depend on any specific properties of ammonia or ammonium ion. The decrease of $H_{II}$ at high pH is probably due to interference with the hydrogen-ion

supply and is attributed, for the present, to reaction (11). This terminal decrease does not follow a simple dissociation curve. Presumably the influence of the ammonia buffer in the reaction layer complicates the relationship.

Numerous attempts have been made to reconstruct the pH curves by combining (*a*) a dissociation curve of maximum height equal to $i_a$ with (*b*) one or more $\beta$ curves or $\beta$ curves which were raised to a power. Limited success has attended these efforts, but the work has suggested a superior technique for studying the effect of pH on the protein curves. In Fig. 10 the heights of waves I and II as reported in section A have been plotted against the product $[NH_4^+][NH_3]$. The product is proportional to the $\beta$ value of an ammonia buffer of total molarity equal to unity (Van Slyke, 1922). Such a plot of a family of curves over a wide range of pH would give the response of the protein waves to variations of pH when the buffering power of the system is constant. Such a technique also circumvents the question of ionic strength which was not controlled in the present study.

## SUMMARY

1. An investigation has been made of (*a*) the effect of electrolyte pH upon the first two polarographic protein waves of five crystalline proteins when the total molarities of the ammonia buffer and cobalt were maintained constant, and (*b*) the effects upon the three polarographic protein waves of crystalline bovine plasma albumin of varying the concentrations of ammonia and of ammonium ion at nearly constant electrolyte pH.

2. Under the conditions of 1 (*a*), the wave heights rose when the electrolyte pH was raised from about pH 3·5 to 8·5 if bivalent cobalt was used. When trivalent cobalt was used the wave heights in neutral and acid media ($i_a$) were quite constant. It is concluded that the enhancement in bivalent cobalt solutions is due to the concomitant increase in concentration of cobaltous hexammine ion.

3. There is a strong correlation between $i_a$ and the total potential sulphydryl content of these proteins. This is the first time that a quantitative relationship has been shown between protein waves I and II and the amino-acid composition of a protein.

4. A satisfactory explanation of this relationship cannot be offered, but it implies that all the potential sulphydryl groups of the protein may be reactive in acid media.

5. In media poised at neutral or acid pH, the pH of the solution layer surrounding the mercury drop (the reaction layer) is probably more alkaline than the bulk of the electrolyte. In a trivalent cobalt solution the pH of the reaction layer is indicated by the pH wave height curve of a particular protein.

6. The protein waves are enhanced when the pH of the electrolyte is in the neighbourhood of the pK of the buffer system and they attain a maximum value on the alkaline side of the buffer pK. At high pH the waves are depressed.

7. The pH at which the maximum wave height occurs seems to depend upon the tyrosine content and the arginine plus lysine content of the protein. The magnitude of the maximum wave height is related to the total potential sulphydryl content of the protein but the relationship is not linear.

8. Certain evidence is tentatively interpreted to mean that at high electrolyte pH the wave height is limited by the dissociation of sulphydryl groups.

9. Under the conditions of 1 (*b*) waves I and II are enhanced (*a*) when the total molarity of the buffer is increased, or (*b*) when the concentration of one of the buffer components is maintained constant and the other is increased. Under these conditions the wave heights are independent of pH in the range 8·7–9·5.

10. Brdička's observations on the effect of pH on wave III, the 'prenatrium' wave, have been confirmed for conditions of low buffer molarity. The effect seems to be more complex when the buffer concentration is high.

11. The reactions and equilibria responsible for the effects are discussed, emphasis being laid on the importance of cobaltous hexammine ion in the catalytic reactions.

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# The Effect of Depressants on the Metabolism of Stimulated Cerebral Tissues

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Depression of the activity of the central nervous system by agents such as barbiturates* is accompanied by metabolic changes in the brain itself. The changes include a fall in respiration, lactic acid, and inorganic phosphate and a rise in phosphocreatine. In investigating the action of depressants, various attempts have been made to reproduce these changes under conditions simpler than those of the whole animal. The metabolic characteristics named can all be measured in cerebral tissues *in vitro*. However, none of the changes brought about in them by depressants *in vivo* has yet been unequivocally reproduced in cerebral tissues *in vitro* by concentrations of the depressants used therapeutically.

Thus, when change occurs in the aerobic formation of lactic acid, it is in opposite senses under the two conditions. It is unchanged or increased *in vitro* by a barbiturate (Hutchinson & Stotz, 1941), by chloral and butobarbitone (Buchel & McIlwain, 1950*a*, *b*; Rosenberg, Buchel, Etling & Levy, 1950) and by pentobarbitone, amylobarbitone, chloretone, tribromoethanol and phenobarbitone (Webb & Elliott, 1951). It is decreased *in vivo* by phenobarbitone, pentobarbitone, amylobarbitone and ether (Stone, 1938; Richter & Dawson, 1948). Phosphocreatine in the tissue also changes in opposite senses *in vivo* and *in vitro*. It is raised *in vivo* during the action of pentobarbitone (LePage, 1946; Dawson & Richter, 1950), but falls *in vitro* during the action of butobarbitone, allobarbitone, and chloral (Buchel & McIlwain, 1950*a*, *b*). Similarly opposed changes take place in the inorganic phosphate of the tissue (Buchel & McIlwain, 1950*a*, *b*).

Observations on respiration come closest to showing parallelism *in vivo* and *in vitro*. They have constituted a large proportion of biochemical work on narcotic action since the demonstration by Warburg (1910) (see also Minami, 1923) that such substances can inhibit respiration. Concentrations of depressants higher than those used therapeutically lower the respiration of cerebral cortical preparations *in vitro* (Loebel, 1925; Quastel & Wheatley, 1933; Quastel, 1951; for an assessment, see Butler, 1950; Himwich, 1951). This, however, is true also of certain barbiturates (Fuhrman, Martin & Dille, 1941) and other agents (Hutchinson & Stotz, 1941) which are not depressant but convulsant. Concentrations comparable to those which are depressive *in vivo* may slightly stimulate (Westfall, 1949, phenobarbitone), depress (Schueler & Gross, 1950, pentobarbitone; Wilkins, Featherstone, Schwidde & Brotman, 1950, phenobarbitone) or be without action on (Zorn, Muntwyler & Barlow, 1939, phenobarbitone) the respiration of cerebral cortex *in vitro*.

In considering possible reasons for the divergence between biochemical findings *in vivo* and *in vitro*, the state of activity of the cerebral tissue appears to be an extremely relevant matter which has not hitherto been explored. The action *in vivo* of depressants such as the barbiturates is accompanied by changes in the electrical activity of the cerebral cortex (see, for example, Brazier & Finesinger, 1945, and the review of Wikler, 1950). Slices of mammalian cerebral cortex *in vitro* do not appear to show electrical signs of activity (McIlwain & Ochs, 1952). Metabolic changes corresponding to those found *in vivo* in association with increased cerebral activity can, however, be induced in such slices by applying suitable electrical impulses to them (McIlwain, 1951*a*, *b*). Metabolic changes, in many ways comparable, can be induced also by increased concentrations of potassium salts (Ashford & Dixon, 1935; Dickens & Greville, 1935; McIlwain, 1952*b*; Gore & McIlwain, 1952), and by 2:4-dinitrophenol (McIlwain & Gore, 1951; see Dodds & Greville, 1933). It was, therefore, decided to examine the effects of some depressants of the central nervous system upon separated cerebral tissues while they were being subjected to the effects of these agents.

## EXPERIMENTAL

Guinea pigs or rats were stunned by a blow on the neck, the brain removed and slices or chopped tissue were prepared from the cortex of the cerebral hemispheres as described by McIlwain (1951*a*, *b*).

The phosphate-glucose and bicarbonate-glucose salines, electrode vessels and tissue-holding electrodes were those

* The following trivial names are used: butobarbitone for 5-butyl-5-ethylbarbituric acid (Soneryl); pentobarbitone for 5-ethyl-5-(1-methylbutyl) barbituric acid; amylobarbitone for 5-ethyl-5-(3-methylbutyl) barbituric acid (Amytal); phenobarbitone for 5-ethyl-5-phenylbarbituric acid; allobarbitone for 5:5-diallylbarbituric acid (Dial).

described by McIlwain, 1951*a*, *b*). Stimulation was by condenser pulses (McIlwain, 1951*a*), which in all cases were alternating and at the frequency of 100/sec., and had the other characteristics described in the individual experiments below. Respiration was measured manometrically and lactic acid by determination according to Barker & Summerson (1941).

## RESULTS

Results are given below with respect to three barbiturates and chloral. As magnesium salts in narcotic concentrations have already been studied by the present methods (Gore & McIlwain, 1952), depressants of three very different types have now been examined. The present agents were chosen to include some of practical utility and some on which considerable biochemical data were already available. Phenobarbitone, butobarbitone and allobarbitone represent aromatic, aliphatic and unsaturated barbiturates with some variations in their speed and type of action *in vivo*. Concentrations of chloral and butobarbitone were chosen which, in the observations of Buchel & McIlwain (1950*a*, *b*), McIlwain & Buchel (1951), Buchel-Olszycka (1947) and Olszycka (1938), had little or no action on the unstimulated respiration of guinea pig and rat cerebral cortex *in vitro*. Similar data with respect to phenobarbitone were drawn from Butler & Bush (1939) and Westfall (1949). Chloral, in these as in other experiments, presumably acts partly after its conversion to trichloroethanol (Butler, 1948, 1949).

### *Effects on electrically stimulated metabolism*

*Respiration.* The course of respiration by normal and stimulated cerebral cortex is shown in Fig. 1 and many such experiments are summarized in Tables 1 and 2. In these, two intensities of stimulation have been used, one submaximal and the other somewhat above the minimum usually required for maximal stimulation.

Fig. 1 shows immediately that phenobarbitone has a markedly greater effect on the stimulated than on the unstimulated respiration of the tissue. The concentration of phenobarbitone used had only a negligible effect in the absence of stimulation. It considerably reduced the effect of the applied impulses at both of the voltages examined.

In these experiments the effects of the stimuli in presence and absence of the drug were examined in different vessels which in initial experiments were also attached to different, individual, stimulating units. Experiments at this stage therefore run in pairs, so that vessels and stimulators which in one experiment were used for following the effect in absence of drugs, were in the next used with the drug. The experiments of Table 1 and of Fig. 1 with phenobarbitone form such a pair. The difference is clearly due to the drug and not to the apparatus. Table 1 also shows that butobarbitone and chloral cause greater inhibition of respiration of stimulated than of unstimulated tissues. The effect with butobarbitone was shown in a glycylglycine- as well as in the usual phosphate-buffered saline, and was found in basal ganglion tissue as well as in cerebral cortex.

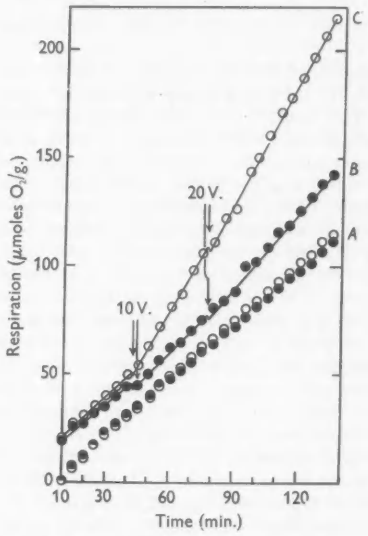


Fig. 1. Effect of phenobarbitone on respiration of stimulated and unstimulated fragments of slices of guinea pig cerebral cortex in salines in vessels *E*. ○—○, glucose-phosphate saline only; ●—●, with $6{\cdot}8 \times 10^{-4}$ M-phenobarbitone. Unstimulated *A*; stimulated *B* and *C*. Stimulation by condenser pulses, time-constant 0·3 msec., as indicated by arrows. Ordinates for the experiments with stimulated material (*B* and *C*) increased by 20 μmoles/g. for clarity. Abscissae give times after placing the vessels at 37°.

The action of some depressants on the respiration of slices of cerebral cortex is shown, in papers quoted above (see also Jowett & Quastel, 1937), to change to a small degree with time, though this was not marked in the present experiments (see Fig. 1). In the experiments of Fig. 1 and Table 1 periods of stimulation always followed an unstimulated period. However, parallel experiments were run in every case in which similar tissues, with and without the drug, were unstimulated throughout. In the calculation of Table 1 the actions of the drugs have been compared during the same experimental period.

Results of adding depressants during respiration are quoted in Table 2. This arrangement enabled the effect of a stimulus from the same source and on the

Table 1. *Action of depressants on respiration and glycolysis of electrically stimulated cerebral preparations*

(Experiments were under the following conditions except when indicated otherwise in the first column. Slices of guinea pig cerebral cortex of 60–70 mg. fresh wt. and cut to about twenty pieces were suspended in 3·5 ml. of phosphate-glucose saline in vessels *E*. In one experiment rat slices were used. The manometric experiments began 23–30 min. after death of the animal and comprised successive periods (indicated) in which the tissues were without impulses, then with impulses at peak potentials of 10 V., then of 20 V.; readings were taken each 5 min. Half the vessels were without impulses throughout the experiments; half contained the drug, which was present in the appropriate media before the tissues were added.)

| Drug (M × $10^{-4}$) | | Metabolism in vessels without stimulation: Respiration (μmoles/g./hr.) 0–30 min. | 30–60 min. | 60–120 min. | Lactic acid (μmoles/g./exp.) | Metabolism in vessels with stimulation: Respiration (μmoles/g./hr.) 0–30 min. (unstimulated) | 30–60 min. (at 10 V.) | 60–120 min. (at 20 V.) | Lactic acid (μmoles/g./exp.) | Change (%) caused by drug in: Respiration Unstimulated | Respiration Stimulated 10 V. | Respiration Stimulated 20 V. | Lactic acid Unstimulated | Lactic acid Stimulated |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Phenobarbitone | 0 | 61 | 56 | 56 | 46 | 59 | 104 | 112 | 145 | — | — | — | — | — |
| | 6·8 | 60 | 56 | 55 | 56 | 58 | 81 | 92 | 122 | −1 | −22 | −18 | +22 | −16 |
| | 17 | 55 | 54 | 54 | 95 | 55 | 62 | 68 | 144 | −9 | −40 | −30 | +104 | −1 |
| Butobarbitone | 0 | 73 | 73 | 73 | 66 | 75 | 90 | 109 | 136 | — | — | — | — | — |
| | 6·4 | 68 | 68 | 66 | 98 | 70 | 76 | 81 | 164 | −7 | −15 | −26 | +48 | +20 |
| Butobarbitone | 0 | 75 | 75 | 75 | 105 | 76 | 112 | 112 | 139 | — | — | — | — | — |
| (in glycylglycine) | 6·4 | 70 | 70 | 70 | 134 | 69 | 97 | 92 | 149 | −8 | −14 | −18 | +28 | +7 |
| Butobarbitone | 0 | 24 | 24 | 21 | 32 | 23·5 | 32·5 | 46·5 | 56 | — | — | — | — | — |
| (basal ganglia chopped) | 6·4 | 26 | 23 | 20 | 46 | 22·5 | 33 | 37·5 | 74 | 0 | 0 | −20 | +44 | +32 |
| Chloral (rat) | 0 | 74 | 74 | 74 | 51 | 70 | 118 | 154 | 139 | — | — | — | — | — |
| | 8·6 | 74 | 74 | 74 | 73 | 70 | 109 | 123 | 127 | 0 | −8 | −20 | +42 | −9 |
| Chloral | 0 | 60 | 60 | 59 | 34 | 59 | 95 | 101 | 129 | — | — | — | — | — |
| | 17 | 54 | 54 | 54 | 56 | 54 | 73 | 80 | 103 | −10 | −23 | −21 | +64 | −20 |

Table 2. *Addition of depressants to respiring cerebral cortex*

(Drugs were not in contact with the tissue until after the first period of stimulation, when they, or saline, were tipped from a side arm. Experiments comprised successive 30 min. periods (*a*) without impulses; (*b*) with impulses at 20 V.; (*c*) with impulses at 20 V. in presence of drug; (*d*) without impulses but in presence of drug. Other conditions were as in the experiments of Table 1. Initial, unstimulated rates were of 62–68 $\mu$moles/g./hr. Values marked with an asterisk (*) are derived from experiments in which no impulses were applied, and periods at similar intervals after commencing metabolism were compared in two different vessels. Other values were derived from rates at different intervals after commencement, but in the same vessel; period *a*/period *d*, or period *b*/period *c*.)

| Solution tipped | Concn. in medium after tipping ($M \times 10^{-4}$) | Change in respiratory rate (%) following addition of drug | |
|---|---|---|---|
| | | Periods without impulses | Periods with impulses |
| Saline | — | 0, −5, 0, −1 | −4, −8, −2, −2 |
| Chloral | 4·3 | +4 | −18 |
| Chloral | 43 | −23 | −52 |
| Butobarbitone | 6·8 | 0, 0* | −18 |
| Butobarbitone | 10 | −4, +12, −17, +5<br>+10*, −12*, +3*, −9* | −34, −11<br>−28, −23 |
| Butobarbitone | 20 | −14, −30, −17* | −38, −34 |
| Allobarbitone | 4·3 | +9, +5 | −20, −27 |
| Allobarbitone | 43 | −21, −23 | −40, −44 |

same sample of tissue to be observed in the same vessel in the presence and in the absence of the drug. Again, greater inhibition of stimulated than of unstimulated respiration was found. In a given experiment the periods with drugs necessarily

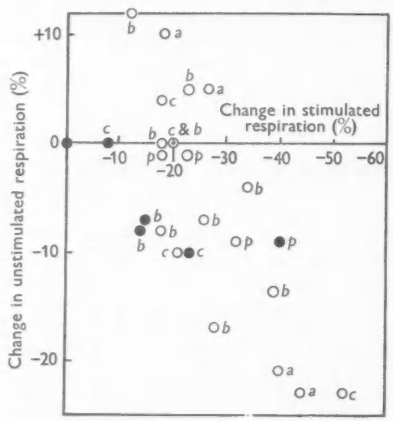


Fig. 2. Comparison of the change brought about by depressants in stimulated and unstimulated respiration of guinea pig cerebral cortex. Stimulation by condenser pulses at 10 V. (●) and 20 V. (○). *a*, allobarbitone; *b*, butobarbitone; *c*, chloral; *p*, phenobarbitone.

followed those without. To control possible differences arising from this, the effect was determined of adding, in place of the drugs, the same volume of saline. The second stimulated and unstimulated periods then showed slightly lower rates than the first, but the changes were very much smaller than those following addition of the drugs.

Direct comparison between the effect of the various agents on stimulated and unstimulated metabolism is made in Fig. 2 by plotting points with these values as co-ordinates. This suggests a relationship between the two effects, and emphasizes that a mean inhibition of some 20% in stimulated respiration is attained without any mean inhibition of unstimulated respiration. The data available do not show effects specific to the different agents. Averaging all values of Fig. 2 shows that a mean inhibition of 5% in unstimulated respiration was associated with a mean inhibition of 18 or 22% (10 or 20 V. impulses) in stimulated respiration.

Corresponding values for Table 2 were −5 and −29%, while saline alone caused changes of −2 and −3·5%.

*Lactic acid formation.* During the experiments described above, lactic acid accumulated in reaction mixtures with unstimulated tissue at a mean rate of about 20–30 $\mu$moles/g./hr. The rate was increased by the depressants, and greatly increased by electrical stimulation.

The impulses, however, always had a lesser effect in the presence of depressants. This is shown in the values given in Table 1. Thus, in the first series of experiments with phenobarbitone, the increase following stimulation in the absence of drug was 99 $\mu$moles/g./experiment, while with the two concentrations of the drug the increases were 66 and 50 $\mu$moles. This might have been due, in part, to the existence of an upper limit to the rate of lactic acid formation by the tissue, and to this being already approached by the stimulus in the absence of the depressant. However, in four of the experiments of Table 1, the lactic acid formed with depressant and stimulus is less in total quantity than that formed with the stimulus alone. This is true also of the

experiment of Fig. 3 in a different medium. This is in spite of the opposite effect of the depressant on the accumulation of lactic acid in the absence of applied impulses.

An impression of the course of formation of lactic acid during such experiments is given by Fig. 3. The ordinates here give the change in pressure in vessels with tissue stimulated and unstimulated in a bicarbonate-containing glucose-saline. The increase of pressure in such experiments with cerebral cortex is almost entirely due to the formation of lactic acid (see McIlwain & Grinyer, 1950; McIlwain, Anguiano & Cheshire, 1951). Fig. 3 shows well, in the upper two curves, how the effect of the phenobarbitone on unstimulated glycolysis is reversed during stimulation.

*Promptness and reversibility of action.* Depressants were added during the measurement of respiration in the experiments of Table 2. The manometric readings from which respiratory rates were derived were taken each 5 min. before and after addition. The lower rate recorded in the presence of the depressant appeared to be established without delay. The accuracy of the measurements was such that this implied the depressant to become fully effective within 3 min. after its addition.

The experiments of Table 3 were carried out to see whether the effect of phenobarbitone on the tissue on which it had been acting would be lost when most of the drug was removed. Tissues were held in the vessel in a grid electrode in salines with and without the drug. After initial periods of metabolism without and with impulses, vessels were removed from manometers and their salines replaced by fresh salines without phenobarbitone. This was done in all vessels, for it was found that replacement of saline lowered metabolic rates in subsequent periods even when the nature of the saline was not changed. When, however, salines containing the depressant were replaced by ones without, stimulated respiration rose. This effect of the drugs is therefore, like their actions *in vivo*, reversible.

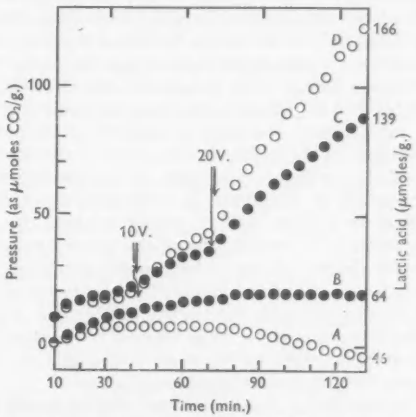


Fig. 3. Effect of phenobarbitone on glycolysis of stimulated and unstimulated fragments of slices of guinea pig cerebral cortex in salines in vessels *E*. ○, glucose-bicarbonate saline only; ●, with $1{\cdot}71 \times 10^{-3}$M-phenobarbitone. Unstimulated *A*, *B*; stimulated *C*, *D*. Ordinates give the pressure changes which were found manometrically, calculated as μmoles $CO_2$/g. tissue; those for *C* and *D* have been increased by 10 μmoles/g. for clarity. Abscissae give times after placing vessels at 37°. The lactic acid found chemically at the end of the experiment is given by the values to the right of the figure.

Table 3. *Reversal of the action of phenobarbitone on electrically stimulated respiration*

(Respiration in each vessel was measured during four periods of 30 min. each, given successively in the table. During the first two of these the vessels were unstimulated, then stimulated; then followed 10 min. without impulses, during which respiration was not measured. The media of all of the vessels were then replaced by fresh salines without phenobarbitone, returned to the thermostat for 10 min., and respiration measured during the final 30 min. periods (without and with impulses). Impulses throughout were at 18 V., applied to slices in the electrodes in vessels *A* (McIlwain, 1951*a*.)

| Initial two periods | | | Final two periods | | | |
|---|---|---|---|---|---|---|
| Respiration (μmoles/g./hr.) | | Increase in respiration due to stimulation (%) | Respiration (μmoles/g./hr.) | | Increase in respiration due to stimulation (%) | Increase in respiration due to stimulation in second period/ increase in first (%) |
| Unstimulated *a* | Stimulated *b* | ($x$ = $100(b-a)/a$) | Unstimulated *c* | Stimulated *d* | ($y$ = $100(d-c)/c$) | ($100y/x$) |
| Without phenobarbitone throughout | | | | | | |
| 48 | 111 | 131 | 40 | 78 | 97 | 74 |
| 56 | 114 | 103 | 51 | 96 | 88 | 85 |
| 62 | 116 | 64 | 60 | 87 | 45 | 71 |
| With phenobarbitone ($1{\cdot}5 \times 10^{-3}$M) in initial two periods only | | | | | | |
| 56 | 87 | 55 | 45 | 90 | 88 | 160 |
| 60 | 96 | 57 | 66 | 105 | 59 | 104 |
| 57 | 66 | 15 | 63 | 84 | 32 | 212 |

*Effect on tissues stimulated with 2:4-dinitrophenol and potassium salts*

Other substances which inhibit the electrically stimulated metabolism of cerebral tissues have been shown to be without action on tissues in which the respiration is stimulated to a comparable degree by 2:4-dinitrophenol or by potassium salts (McIlwain, 1951*c*). The depressants now being studied showed, on the contrary, a relatively uniform action independently of the mode of stimulation of the tissue.

2:4-Dinitrophenol was used in the present experiments at $5 \times 10^{-5}$ M, a concentration found (McIlwain & Gore, 1951) to cause maximal rise in respiration. It caused, during the present experiments (Table 4), an approximate doubling in respiratory rate, the stimulated values being close to those obtainable electrically (Table 1). Both phenobarbitone and chloral lowered these rates by about 25%, when present at concentrations having little effect on unstimulated metabolism.

Accumulation of lactic acid was also increased by 2:4-dinitrophenol, but the effect of depressants on the accumulation was less than that observed during electrically stimulated metabolism. The lactic acid formed during the experiments of Table 4 was throughout less than that formed in those of Table 1; contributing to this were the shorter experimental period, the use of intact slices, and the absence of additional handling of the slices involved in the use of tissue-holding electrodes.

Potassium chloride was used in most experiments in a concentration (0·033 M) giving a large but not maximal increase in respiration, and a small change only in lactic acid accumulation. One set of experiments was also carried out with 0·1 M-potassium salts, which gave maximal effect on respiration and also markedly increased glycolysis. Respiration stimulated with either concentration of potassium salts was markedly inhibited by both phenobarbitone and chloral. The effect on accumulation of lactic acid was relatively small.

Considering all the experiments of Table 4 with both 2:4-dinitrophenol and potassium chloride as stimulants, the mean inhibition of unstimulated respiration by the depressants was 5%, while they inhibited stimulated respiration by 25%. This is very similar to the effects of the depressants on electrically stimulated tissue. The mean changes caused by depressants in lactic acid formation during chemical stimulation were much smaller than during electrical stimulation.

## DISCUSSION

Present studies and previous work suggest for depressants two action types, one on the metabolic behaviour of brain, the other on electrical phenomena. These will be first discussed and then compared.

*Metabolic changes.* New findings are (1) that stimulation of cerebral tissues electrically or otherwise increases the sensitivity of their respiration to depressants; (2) that lactic acid formation in electrically stimulated sections is lowered by depressants. Both findings show that stimulated sections simulate events *in vivo* more closely than do unstimulated ones. Quantitative comparison is as follows. By arterio-venous difference and blood flow in man, Himwich, Homburger, Maresca & Himwich (1947) found cerebral respiration to be little affected by pentothal during the early stages when consciousness was clouded, but to be inhibited some 30% after loss of contact and during surgical anaesthesia. Deep pentothal narcosis in the monkey was observed to be associated with inhibition of respiration by 35% (Schmidt, Kety & Pennes, 1945). In these cases, the concentration of drug in the blood stream or in cerebral tissues was not determined, but Grenell & Davies (1950) investigating perfused cat brain, found $5 \times 10^{-4}$ M-pentobarbitone to inhibit cerebral respiration by 20–25%. This order of concentration and inhibition has been reproduced during the present experiments with stimulated tissues (Tables 1 and 2). As has been indicated throughout this paper, comparisons between the present results and those of other workers frequently involve related, rather than identical, drugs and species.

Preferential action on the more active central nervous system is of obvious advantage in a depressant drug. Such drugs have been selected specifically in order to depress partially and reversibly; not to cause complete loss of function, but to convert a greater to a lesser level of activity. While many types of mechanism other than biochemical are likely to be involved in this, the present findings show that contributions to such selectivity can be made by reason of the different levels of respiratory activity in normal and excited tissue. Depression of the metabolism of electrically stimulated slices was lost when the drug was removed, as is the depression *in vivo*.

Selective inhibition of the stimulated respiration suggests that the factors limiting respiration of slices when their oxygen uptake was at the level of some 120 $\mu$moles/g./hr. were different from those limiting respiration at the level of 60 $\mu$moles/g./hr. It is not known whether the factor in either case corresponds to that stage in respiration of brain homogenates concluded by Greig (1946) to be most sensitive to depressants. It is probable, however, that the higher rate corresponds most closely to the rate of metabolism by cerebral cortex *in vivo*. A mean value for the normal oxygen consumption of the brain of monkeys *in vivo* given by Schmidt *et al.* (1945)

Table 4. *Effect of depressants on respiration and glycolysis of cerebral cortex stimulated with 2:4-dinitrophenol and potassium salts*

(For each of the first four groups of experiments, weighed intact slices from the cerebral hemispheres of a guinea pig were distributed between two sets of vessels, one set containing ordinary glucose-phosphate saline and the other set with saline containing the stimulating agent. Respiration was measured for a period of 45 min., the depressants then added from side arms, and respiration measured for a further 45 min. The depressants added were dissolved in the type of saline (with or without the stimulating agent) present in the main compartment of the vessels. The side arms of the control vessels contained only the corresponding saline, and the lactic acid in these vessels was compared at the end of the experiment with that found in vessels to which drugs had been added. In the last two sets of experiments, marked with asterisks (*), vessels with and without chloral or KCl from the commencement of the experiment were compared; duration 90 min.)

| Added drug | Final concentration ($M \times 10^{-4}$) | Metabolism in media without stimulant: Respiration ($\mu$moles $O_2$/g./hr.) Before addition | Metabolism in media without stimulant: Respiration ($\mu$moles $O_2$/g./hr.) After addition | Metabolism in media without stimulant: Lactic acid ($\mu$moles/g./exp.) | Metabolism in media with stimulant: Respiration ($\mu$moles/g./hr.) Before addition | Metabolism in media with stimulant: Respiration ($\mu$moles/g./hr.) After addition | Metabolism in media with stimulant: Lactic acid ($\mu$moles/g./exp.) | Change (%) caused by drug in mean values for: Respiration Unstimulated | Respiration Stimulated | Lactic acid Unstimulated | Lactic acid Stimulated |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | $5 \times 10^{-5}$ M-2:4-Dinitrophenol | | | | | | |
| Phenobarbitone | 0 | 57 | 54 | 18 | 120 | 120 | 52 | — | — | — | — |
| Phenobarbitone | 11 | 57, 60 | 57, 57 | 17, 21 | 117, 132 | 102, 90 | 47, 45 | −2 | −25 | +6 | −10 |
| Chloral | 0 | 54 | 54 | 20 | 129 | 126 | 52 | — | — | — | — |
| Chloral | 11 | 58 | 55 | 25, 19 | 120, 114 | 90, 96 | 59, 65 | −5 | −26 | +10 | +19 |
| | | | | | 0·033 M-$K^+$ | | | | | | |
| Phenobarbitone | 0 | 60, 63 | 54, 66 | 26, 28 | 96, 96 | 84, 99 | 30, 35 | — | — | — | — |
| Phenobarbitone | 6 | 60, 57 | 66, 57 | 28, 29 | 84, 96 | 66, 78 | 27, 21 | +5 | −20 | +5 | −27 |
| Phenobarbitone | 12 | 60, 62 | 57, 48 | 24, 26 | 102, 102 | 78, 72 | 31, 35 | −13 | −36 | −7 | 0 |
| Chloral | 0 | 66 | 66 | 24 | 81 | 78 | 26 | — | — | — | — |
| Chloral | 12 | 63, 72 | 57, 66 | 26, 20 | 90, 90 | 63, 63 | 24, 27 | −9 | −30 | −4 | +2 |
| Chloral* | 0 | 57 | — | 32 | 84, 87 | — | 38, 35 | — | — | — | — |
| Chloral* | 12 | 54 | — | 30 | 75, 72 | — | 27, 29 | −5 | −14 | −6 | −23 |
| | | | | | 0·10 M-$K^+$ | | | | | | |
| Chloral* | 0 | 66 | — | 40 | 111, 102 | — | 59, 59 | — | — | — | — |
| Chloral* | 12 | 60 | — | 45 | 84, 90 | — | 65, 61 | −9 | −18 | +13 | +7 |

corresponds to 79 μmoles/g./hr. Grey matter from the cerebral cortex respires *in vitro* at twice the rate of cerebral white matter.

Varied effects of depressants on respiration of cerebral preparations were quoted at the beginning of this paper. It is possible that certain such variations may be related to the level of respiration attained under the experimental conditions used. Jowett & Quastel (1937) found phenobarbitone to have a greater effect in the absence than in the presence of calcium and magnesium salts. Westfall (1951) again reported phenobarbitone to inhibit or stimulate according to the content of magnesium and calcium salts in the surrounding medium, and several of these changes were in the sense that a high rate of respiration was depressed, and a low one increased. Schueler & Gross (1950) found inhibition *in vitro* with pentobarbitone at anaesthetic concentrations in whole blood. Rates are not recorded in known units, but are likely to be higher in such a medium than in simple salines (cf. Krebs, 1950). Moreover, in a study published after the present results had been obtained, Webb & Elliott (1951) compared the effects of depressants on the respiration of cerebral cortex slices in presence and absence of added potassium salts. The potassium salts had relatively little action on respiration; no explanation of this divergence from their normal effect could be given. But, associated with the small stimulation of respiration, there was an absence of increased sensitivity to depressants.

*Electrical phenomena.* Of the means used in the present investigation for increasing the activity of cerebral tissue, electrical stimulation is most relevant to circumstances *in vivo*, when the brain shows continuous electrical activity. The inhibition by depressants of electrically induced activity in nervous tissues has frequently been observed, although electrical rather than metabolic means of observing such activity have been more frequently employed.

Thus the threshold to stimulation may be raised or axonal conduction depressed in various elements of the central nervous system, or its spontaneous electrical activity changed in the cerebral cortex (Derbyshire, Rempel, Forbes & Lambert, 1936; Forbes & Morison, 1939; Hoagland, Himwich, Campbell, Fazekas & Hadidian, 1939), the spinal cord (Heinbecker & Bartley, 1940; Bremer & Bonnet, 1948) or in a sympathetic ganglion (Posternack & Larrabee, 1948, 1950). This can occur at anaesthetic concentrations, and particular characteristics measured by electrical methods are affected somewhat differently by different depressants (Swank & Watson, 1949; Finesinger & Brazier, 1944; and some of the preceding references). Moreover, the responses to stimulation as measured by electrical techniques may be prevented at concentrations of depressants lower than those necessary for effects on the resting respiratory activities of the tissue. This was found to be the case in the stellate ganglion of the cat (Larrabee, Ramos & Bulbring, 1950), where pentobarbitone or chloretone depressed synaptic transmission at anaesthetic concentrations which had no effect on the oxygen uptake of the tissue, in absence of stimulation. Depression of these various aspects of functional activity may well be mediated by metabolic effects of the drugs such as those on acetylcholine described by McLennan & Elliott (1951).

Cerebral tissues can respond to a fall in energy-consuming processes by a fall in energy-yielding processes. Schmidt *et al.* (1945), among others, showed that the rate of metabolism of the brain *in vivo* depended on its level of activity; comparable phenomena *in vitro*, and the mechanisms available to establish such connexions have been surveyed elsewhere (McIlwain, 1950, 1952*a*). Thus depression of electrical activity under the circumstances described above would be expected to be associated with a fall in respiration and glycolysis in the excited tissues.

*Possible balance of inhibitions* in vivo. The conclusions arrived at in the preceding two sections are different and complementary. Metabolic findings indicate that respiration, however stimulated, becomes more susceptible to concentrations of depressants active *in vivo*; inhibition of respiration may thus be responsible for the depression of function *in vivo*. The second section indicates that depression of function can lead secondarily to lowered respiration without the depressants acting on any respiratory step.

The question of which, if either, of these processes preponderates *in vivo* can probably be answered from data already available (McIlwain & Buchel, 1951). Energy-rich compounds are observed to accumulate during narcosis *in vivo* and this makes it likely that in the brain as a whole, inhibition of energy-consuming activity is greater than that of energy-yielding processes.

However, inhibition of both processes may be necessary in a successful depressant. Disproportionate inhibition of energy-yielding mechanisms would be expected, as in hypoxia or hypoglycaemia, to be destructive to cerebral tissues. Disproportionate inhibition of energy-consuming mechanisms may also lead to instability. Thus, atropine at $5 \times 10^{-4}$ M has no effect on the respiration of cerebral cortex *in vitro* either in an unstimulated condition or when stimulated by 2:4-dinitrophenol and by potassium salts, but it prevents the response of such tissues to electrical stimulation (McIlwain, 1950). The central effects of atropine (excitement, hallucination, and delirium) are not those of a depressant. A greater or lesser imbalance in inhibition of

energy-yielding and energy-consuming reactions may be responsible for certain barbiturates being convulsant, and for the transitory excitory phase in the action of many depressants.

## SUMMARY

1. Phenobarbitone, butobarbitone, allobarbitone and chloral inhibited the respiration of electrically stimulated sections of rat and guinea pig cerebral tissues. They did so at concentrations below $10^{-3}$ M which had little or no effect on respiration of the same preparations in the absence of stimulation.

2. Stimulation of the respiration of cerebral cortex by potassium salts and by 2:4-dinitrophenol also rendered it more sensitive to depressants. In this, the depressants acted differently from a substance such as atropine which had a selective effect on electrically stimulated tissues.

3. The depressants decreased the lactic acid formed from glucose by electrically stimulated cerebral tissues. This change occurs also during narcosis *in vivo* and is in the opposite sense to that induced by the depressants in unstimulated tissues *in vitro*.

4. Inhibition of the respiration of electrically stimulated tissue was prompt and could be reversed when the drug was removed.

5. It is concluded that a successful depressant acts *in vivo* by inhibiting both energy-yielding and energy-consuming processes in the central nervous system.

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# Techniques in Tissue Metabolism. 1. A Mechanical Chopper

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The apparatus now described was devised to chop small and irregular fragments of animal tissues for metabolic experiments, with as little disruption as possible of cell structure. It will also cut larger pieces of tissue. Small and irregular specimens were being examined because they are often the only ones available at biopsy or when small organs, or parts of them, are being investigated. Cell structure was required to be relatively intact in order to disturb as little as possible the many metabolic characteristics on which such structure exerts a controlling influence. These include levels of metabolites and metabolic rates, concentration or exclusion of added substances, and response to inhibiting and stimulating agents. Several instances of the importance of such structural factors in preparations from the central nervous system have been encountered recently (McIlwain & Grinyer, 1950; McIlwain, Buchel & Cheshire, 1951; McIlwain, 1951*b*). In some of these studies (see also McIlwain, Ayres & Forda, 1952) specimens had been reduced to a size small enough for diffusion of metabolites to the tissue, by a method consisting of 'chopping' free-hand, by repeatedly passing a blade vertically into the tissues. The machine now described was built to do this in a regular and reproducible fashion.

## EXPERIMENTAL

### *Preliminary observations*

At least three different types of treatment have been used to fragment tissues.

*Cutting.* A blade with a relatively small cutting angle is moved both across and into the specimen simultaneously. Its motion is in the plane of the blade and has components both parallel and perpendicular to its cutting edge. Efficacy in cutting does not markedly vary with the speed of motion of the blade.

*Shearing with scissors.* Opposed cutting edges with large cutting angles are made to approach the specimen in directions perpendicular to their cutting edges.

*Chopping.* This motion, as of an axe or of a knife in chopping vegetables, has been applied little in dissection. It is, however, applied to hardened or supported tissues in microtomes. A sharp blade with relatively small cutting angle is used and approaches the tissue directly without necessarily any motion parallel to the cutting edge. The result, when applied to soft tissues, depends greatly on the speed of approach of the blade.

It appeared that the static rigidity of embedded tissues might be simulated by the resilience and inertia which they show, while still fresh, to a sudden blow. If a scalpel about the size and weight of a pocket knife is held by the extremity of its handle and its blade swung against a piece of liver lying on a pad of filter paper, the tissue can easily be cut (chopped) cleanly and suffers little depression at the point of entry of the blade. On the other hand, if the blade is laid on the tissue and pressed directly into it, appreciable force is needed for the cut and the tissue is distorted. (If a cutting motion is given to the blade, then again, much less force is required.)

There appeared to be relatively little advantage, when 'chopping' with a scalpel, in giving the blade an additional motion parallel to the cutting edge, as in ordinary cutting. A simple chopping motion was therefore made the basis of the machine described.

### *Description of the chopper*

Fig. 1, Pl. 1, is a view of the chopper from the position of a person using it, and Fig. 2, Pl. 1, a view from a position at his left-hand side. Fig. 3 gives constructional details and omits the motor and rheostat which are at the back of Fig. 1, Pl. 1.

*Motion of the blade.* Chopping is by the blade *B*, held in arm *A*. This is lifted by the trigger cam *C*1 and then released, when it is drawn by its own weight and by the spring *Sp*1 into the specimen on the cutting table *T*. This is the only motion of the blade; it is lifted and released at each revolution of the shaft *Sh*1.

While the machine is cutting, shaft *Sh*1 is rotated by an electric motor, being continuous with the shaft of a helical

reduction-gear built to the motor framework. A handwheel *H*1 is provided on shaft 1 so that the blade can be lifted if, at the end of a cut, when the motor has been switched off, the blade has stopped in an inconvenient position (in the specimen and not in the air). With a little practice, however, the motor can be switched off at a moment such that the blade remains out of the specimen without further adjustment.

spring *Sp*3 against the large ratchet wheel. The ratchet wheel is thus partly rotated each time the lever rises, and turns with it the shaft *Sh*2. This shaft carries a large lead-screw (1 in. diam., Whitworth, 8 threads/in.) which passes through a corresponding thread attached to the base of the table *T* carrying the specimen. The specimen is thus moved to the right (viewing the machine as in Fig. 1, Pl. 1) at each revolution of shaft 1. The cams are arranged so that the

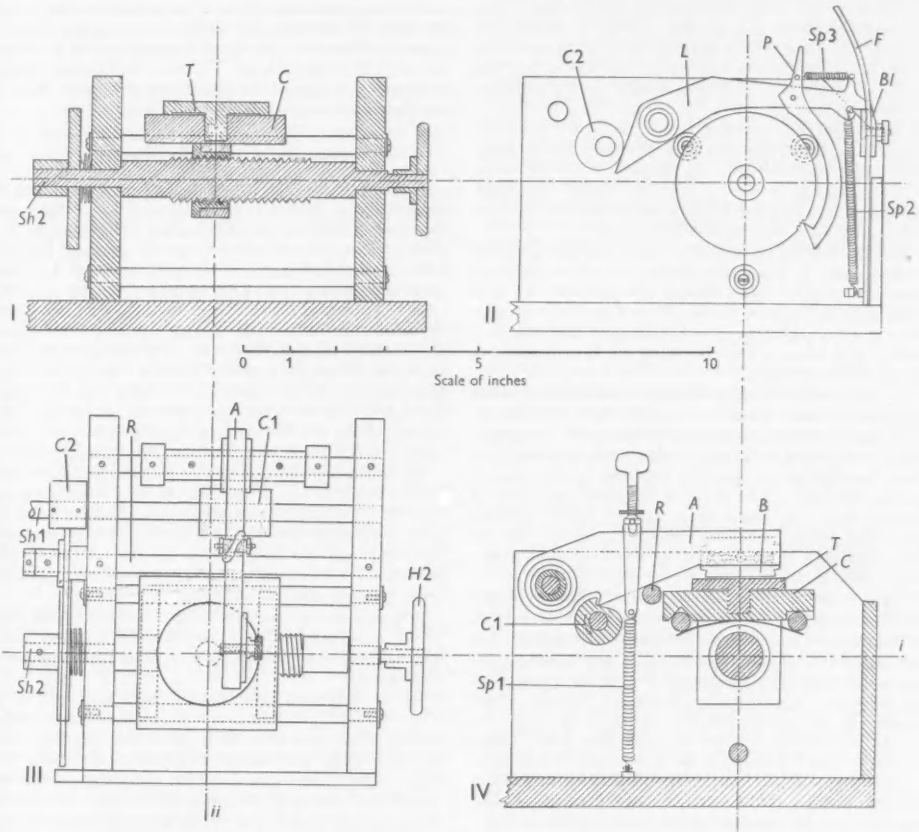


Fig. 3. Constructional details of the workshop-made parts of the chopper. Motor, rheostat, switch and most of the baseboard are omitted. I: sectional elevation through centre line (i), omitting cutting arm. II: end elevation from left-hand side of I, omitting cutting arm and part of frame *F*; a few teeth only are shown on the ratchet wheel. III: plan, omitting baseboard, pawl *P* and frame *F*. IV: sectional side elevation on centre-line (ii) from left-hand side. *A*, cutting arm; *B*, blade; *Bl*, block in frame; *C*, carriage for cutting table; *C*1, trigger-cam; *C*2, cam; *F*, frame within which moves the lever *L* which turns *Sh*2; *L*, lever moved by *C*2; *P*, pawl; *R*, rod on which the cutting arm falls; *Sp*1, spring which draws down the cutting arm; *Sp*2, spring which keeps *L* against *C*2; *Sp*3, spring which keeps pawl against the ratchet wheel; *T*, cutting table on which specimen is placed.

*Motion of the specimen.* Shaft 1 carries, as well as the trigger cam, an eccentric cam *C*2 which operates a lever *L* which is held against it by a spring *Sp*2. At each revolution of shaft 1, the end of the lever opposite to that bearing on *C*2 is raised, and carries with it the pawl *P*. This is held by a

specimen moves only while the blade is withdrawn from it. The blade falls each time in a different part of the specimen, and thus cuts it to a series of slices. This is the second basic movement of the machine. In addition to these movements, certain adjustments are provided.

*Adjustments.* (1) The thickness of the slices cut is governed by the extent to which the specimen moves between each cut of the blade. Provision is made for altering this by limiting the extent which lever *L* moves at each rotation of shaft 1. The end of the lever farthest from the shaft rises and falls within a slit in a frame *F*. The slit carries a block *Bl* which can be clamped in various positions by a screw. The relative positions of the lever and frame are such that, when the block is at its highest point, the movement of the lever is enough to move the pawl over one tooth of the ratchet wheel; but, when the block is at its lowest point, it moves over 14 teeth. The wheel has 120 teeth and thus, at the minimum setting of the block, it moves 1/120 rev. and the table moves $\frac{1}{960}$ in. or 0·0264 mm. At the maximum setting the table moves 0·356 mm.

After a specimen has been sliced, the table is returned manually by lifting the pawl from its ratchet and rotating shaft 2 by a handwheel *H* 2. As the pitch of the leadscrew is large, this can be done reasonably rapidly (the table moved 1 in. in 4 sec.) and other arrangements for returning the carriage were not considered necessary.

(2) The table rotates about a central, vertical pin. The vertical plane in which the specimen is cut is thus continuously variable. After the specimen has been cut to a series of slices, it can be rotated 90° and the slices cut to a series of prisms. For this purpose, there are cut in the rim of table *T* four aligning grooves which can be opposed to a groove in the carriage *C*.

(3) The extent to which the blade descends on its release is governed by contact between the arm *A* and the rod *R*. These are not adjustable, but the blade (an ordinary double-edged safety-razor blade) can be adjusted in relation to the arm. It is held to the arm by a single screw which passes through the centre hole or slit of the blade and carries a knurled nut. This bears on a strip of fabric bakelite, 4·5 × 2 cm., which is shaped to a shallow arc so that when the nut is tightened by the fingers, the blade is gripped over a large part of its surface, against the arm *A*.

The position of the blade in the arm is chosen as follows. Three moist filter papers are placed on the table, a blade loosely clamped to the arm, and the arm brought to rest against rod *R* by rotating handwheel 1. The blade is pressed by the fingers so that it is in contact with the paper along the whole of its lower cutting edge and clamped in this position by the knurled nut. The machine is then switched on. It will probably just cut the upper filter paper. This is removed. That below will probably be intact and ready to receive a specimen. During the cutting of a series of specimens, it may be necessary to add or remove a filter paper to bring the specimen to its correct height.

(4) The speed and force with which the blade descends can be adjusted by the spring 1. This is connected to a bridle with an adjustable screw. Suitable springs for use in this position were found empirically, and the following measurements were then made by temporarily attaching a spring balance to the arm at a point corresponding to the centre of the blade: to lift the blade without an attached spring, 50 g.; to lift the arm, while minimum tension was applied by a light spring, 200 g.; to lift it while maximum tension was applied to the same spring, 300 g.; to lift it while maximum tension was applied to a stronger spring, 650 g. The lighter spring has been used in most of the work described in this paper.

The blade falls a distance of 1·6 cm. at its centre point. The shape of the eccentric cam and its relationship to the arm and shaft 2 are such that after the fall of the blade, shaft 1 turns 45° before commencing to lift the arm. With the light spring at its minimum tension, the arm just failed to strike rod *R* when shaft 1 was running at 220 rev./min. The 1·6 cm. was, therefore, covered in 0·034 sec.; average velocity, 47 cm./sec. Assuming uniform acceleration, the final velocity was thus about 95 cm./sec. With the spring 1 at a maximum tension, this rose to about 150 cm./sec.

(5) A rheostat connected in series with the motor gave continuous variation in speed. A normal speed of operation has been 150 cuts/min. The machine can operate at more than twice this speed. The speed of operation does not affect the speed of fall of the blade, but does affect its rate of rise. Presumably because of this, a tissue which tends to stick to the blade is most successfully cut at slower speeds.

### *Manner of using the chopper*

*Small specimens.* The specimen of tissue, or a portion of suitable size cut from it, is weighed on a torsion balance and placed on a sheet of hard filter paper (Whatman no. 50) already moistened with saline by quickly passing it through a dish of saline and sharply shaking off the excess. The filter paper and tissue are placed on the table of the machine. The table has previously been moved to the left of the blade, the blade adjusted in height, and the ratchet arm set to give the necessary thickness of section. The machine is switched on so that it cuts the specimen to strips. At this point the specimen can be removed, or the table can be turned through 90°, the specimen again brought to the left of the cutting blade, and the machine again switched on. After cutting, it is stopped with the blade out of the specimen.

The paper and chopped specimen are removed from the table, the paper held in the hand and the specimen lifted from it with a spatula moist with a drop of saline. When the specimen is on the spatula, the saline is allowed to drain into the paper and the specimen on the end of the spatula transferred to the experimental vessel, usually by shaking it under the fluid which is being used.

A typical size of specimen handled in this way, has been 15–200 mg. No difficulty has been found in transferring the whole of such specimens, after chopping, with a stainless-steel spatula.

*Larger specimens.* Specimens of several tissues up to 3 × 3 × 0·5 cm. or about 4 g. in weight, can be cut in a single loading of the machine. These have been handled either (*a*) by weighing beforehand and transferring entirely to a single experimental vessel, (*b*) by transferring to several vessels and weighing the tissue afterwards, or (*c*) distributing volumetrically, if the material permits handling as a suspension. In this case, either the whole or a portion can be weighed.

Distribution volumetrically can be as successful with chopped tissue as with the mince from a Latapie machine, but is not as successful as with the suspension from a Potter-Elvehjem homogenizer. It has been most successful with tissue placed on the table of the machine as a block not more than 2 mm. in thickness, and then cut to prisms not more than 0·2 × 0·2 mm. square in section. A pipette with a wide orifice close to the bulb has been employed.

*Handling specimens before and after chopping.* Little if any pretreatment is needed for many tissues, but the following three points should be noted: (1) A tough sheath of markedly different consistency from the rest of a specimen should be removed; e.g. that around the kidney, although equally

tough material such as skin can be successfully chopped alone. (2) The simplicity of operation with most tissues depends on their adhering to moist filter paper and not to the chopping blade. This can be aided in various ways. Passage of the blade is facilitated by moistening the blade or the top of the specimen with a camel-hair brush previously dipped in saline and squeezed between filter paper to the desired extent. It is best to moisten most specimens in this way. (3) An approximately flat surface is required on the specimen to cause it to adhere to the filter paper on the cutting table. Thus, a kidney must be cut in half and the portions laid on the cut surfaces, but a lobe of liver needs no preparation.

After cutting a specimen and transferring it to fluid, the individual fragments, although completely cut, need shaking or stirring to separate them. The shaking normally given during a metabolic experiment may be sufficient for this. Separation may also be done by the spatula used in transference, by jerking the vessel containing the fluid and specimen, or by holding the vessel for a second or so against the moving rubber-covered jaws of a Microid flask shaker or similar agitator.

### *Notes on construction and components*

The base which is 14 in. (35 cm.) square has four rubber cushions on its under surface. It and the two main uprights which carry all shafts, are of $\frac{3}{8}$ in. (1·6 cm.) bakelized paper. The shaft holes are close running fits without bushes. Shaft *Sh* 2 which moves the carriage is held to the left (Fig. 3, I and III) by a flat helical spring. The carriage is guided on two rails *R* of round silver steel, by a V and flat machined in its under surface. It is held to the rails by flat springs of phosphor-bronze. The lever *L* and the pawl are of fabric bakelite and are held by springs at tensions of respectively 150 and 250 g. The cutting arm is also of bakelite, and the cams and ratchet wheel of brass. The cutting arm must be rigid and not tend to vibrate. For this reason it has long bushes (see Fig. 3, III) which are close fits on the shaft on which it pivots, and the arm itself is $\frac{3}{8}$ in. (0·95 cm.) thick.

The motor is a universal series-wound type of $\frac{1}{3}$ h.p., built to run at its driving shaft (after a reduction gear), under load, at 300 rev./min. with a torque of 9 lb. in. Its speed is controlled by a resistance (280 Ω) in series with the motor. Also in series with the motor is a two-pole switch mounted on the front panel and seen in Figs. 1 and 2, Pl. 1. The model of motor shown in these photographs may not be generally available, but an equivalent is Type SA 90-W 50 supplied by Fractional H.P. Motors Ltd., London, N.W. 9. (this would require a resistance of 490 Ω).

Ordinary safety-razor blades with two cutting edges of 3·7 cm. were used, after removing their waxy coating. Each edge sufficed for several thousand chopping blows, or for cutting some scores of specimens, without deteriorating. They were kept from day to day by drying with filter paper after use. In practice, a new blade has been used every few days as their cost is small. Stainless-steel blades, kindly given by Prof. A. St G. Huggett, have the advantage of being packed without wax and probably last longer. Blades which are thicker than the type illustrated in Fig. 3, IV, and which project farther from the cutting arm, can be made from strips of blading supplied by manufacturers of the types of safety-razor blades which have single cutting edges.

In an earlier model of the machine, the blade was given an oscillating motion by a driving wheel and a link mounted eccentrically on it. This proved adequate for cutting certain tissues only; the downward speed of the blade was dependent on the speed of operating the machine as a whole, and was minimal at the base of the specimen.

### *Metabolic and electrical techniques*

In the examples quoted in the tables, conventional manometric apparatus was used with the following additions. Electrode vessels were pattern *E* of McIlwain (1951*b*). Electrical stimulation was by diphasic condenser pulses, 100/sec., of time-constant 0·35–0·5 msec. (McIlwain, 1951*a*) from a mains-operated stimulator (McIlwain & Ayres, unpublished). The glucose-phosphate and glucose-bicarbonate salines were those described by McIlwain (1951*a*). Experiments were run at 37°, in $O_2$. Tissues were obtained and prepared as described by McIlwain (1951*a*), and McIlwain *et al.* (1952), and were mostly from guinea pigs and rats. We are greatly indebted to Mr Murray Falconer, Director of the Guy's-Maudsley Neurosurgical Unit, for specimens of human cerebral tissues which, though removed for therapeutic purposes, were macroscopically normal.

## RESULTS

### *Qualitative observations*

Most animal tissues and many organs were cut with the machine and yielded preparations suitable for metabolic work. Tissues satisfactorily cut included spinal cord, which is soft; skin, which is tough; and lung which has a loose texture and fibrous elements. Brain, liver, kidney and heart muscle presented no difficulties.

*Central nervous system.* Specimens from the brain were among the easiest to cut with the machine. No preparation, apart from choice of the area to be cut, was necessary with specimens from small laboratory animals, but membranes and blood vessels closely adhering to the surface were removed from human specimens prior to chopping. White and grey matter were cut together in the same specimen without distortion or tendency to separate. This can be seen in the cerebellar slices included in Pl. 2. Slices made by setting the machine at 0·21 mm. thickness of cut are remarkably uniform in thickness. This can be seen in the photograph of cerebral cortex, chopped in two directions at right angles and with this thickness of cut. The initial specimen in this case was about 3 mm. thick, so that it yielded prisms $0{\cdot}21 \times 0{\cdot}21 \times 3$ mm., of which the long sides were formed entirely by the cutting. They are seen to be uniform. Each prism carries at one end a portion of the subcortical white matter. Although the cut tissue has been shaken with moderate vigour to separate its individual sections, these have not markedly fragmented.

Specimens were cut equally well in planes bearing various relationships to the direction of the fibres they contained. Thus spinal cord, after removing its sheath, could be prepared for cutting as disks of

2 or 3 mm. in thickness made by cross-sectioning, and then chopped parallel to the direction of the main fibres; or 1 or 2 cm. of the cord could be split longitudinally by extending the medial fissures, laid on the table on its cut surfaces and chopped perpendicularly to the fibres. Portions of the brain stem of a guinea pig could be dissected out, weighed, chopped and transferred without loss to an experimental vessel. So also could the brain of a mouse, or the complete cerebral hemisphere of a guinea pig.

The downward speed of the blade was not found critical in cutting specimens from the central nervous system. Such specimens could also be cut with the earlier model of the machine. Quantitative data are given below regarding the metabolic behaviour of chopped cerebral tissues.

*Liver.* Specimens from any position in the organ were chopped to slices or prisms by cuts at least as small as 0·2 mm. When a worn blade was used, it was evident that material from the edge of a lobe cut more easily than from the centre, where vascular or fibrous elements were larger. In material from laboratory animals these presented no difficulty. Their presence, however, made the slices or prisms less regular than those obtainable from cerebral tissues. This is shown in Fig. 6 (Pl. 2), in which chopped guinea pig liver is clearly not so uniform as is chopped cerebral cortex. The specimen was seen to spread during chopping so that it occupied a slightly larger area on the cutting table after the chopping than it did before.

A complete lobe of guinea pig liver without initial preparation (some 3 × 2 cm. and 3 g. in weight) was accommodated on the table and chopped at 0·32 mm. intervals in two directions in 1 min.

*Spleen.* This behaved much as did liver.

*Kidney.* A kidney of up to 2 g. from a guinea pig or smaller animal was accommodated in the machine. It was prepared by removing the transparent outer sheath, cutting in half by a longitudinal cut through the hilus, and placing on the paper of the machine with the cut surfaces downwards. When so prepared, the medulla was chopped nearly as cleanly as the cortex. This is very difficult to achieve by cutting (as distinct from chopping), with a blade either free-hand or with a template. When the medulla was freed from cortex before chopping, it tended to be picked up by the blade. Cortex alone presented no difficulty. Fig. 7 (Pl. 2) shows slices obtained by chopping a portion of guinea pig kidney. The majority, but not all, are complete cross-sections of the specimen. When chopped to prisms, these were of a regularity intermediate between those shown for cerebral cortex and liver.

*Adrenal.* Outer sheaths were removed from guinea pig or rabbit adrenals, they were cut in half along their largest plane and placed on the cutting table on the cut surfaces. Sections of 0·2 or 0·3 mm. were made successfully and included both the cortex and medulla. If they were suspended in saline and shaken strongly, the medulla dispersed. Glands were also cut on several occasions by laying them on their largest flattish surface, without preliminary section.

*Lung.* Portions of guinea pig lung, without preparation, were cut to 0·2 or 0·35 mm. slices, but these tended to remain connected by fine fibres in that part of the specimen which had been applied to the filter paper on the cutting table. They could then be detached by scissors or a scalpel. The tendency to remain attached could be minimized by allowing the blade to chop through not only the specimen but also a layer of the filter paper on which it was placed. When part of the outer surface of the specimen, especially the angles running the length of the lobes, was removed with scissors before chopping, the attachment was minimized. Slices obtained by chopping were much more regular than can normally be prepared from lung by cutting with a blade.

*Heart.* The muscular wall of the heart, taken from various positions, was cut satisfactorily to sheets 0·2 or 0·35 mm. in thickness. The sheets curled after suspending in salines, but not to such an extent as would be expected to hinder diffusion to their interior.

*Abdominal wall.* The specimens examined were 2–3 mm. in thickness with two main layers between which lateral motion could occur. No difficulty was encountered in cutting them with a fairly fresh blade. After suspending in saline, the strips curled.

*Skin.* This offers a good example of how qualitative observation with a swung scalpel (see p. 412) agrees with the performance of the machine. Skin lying on moist filter paper is relatively difficult to cut with a scalpel in the ordinary way, unless tension is applied to the skin, but it can easily be cut with a sharp blow of the scalpel. For cutting in the machine, skin from the neck or abdomen of a guinea pig was plucked free of most of its hairs, cut out with scissors at approximately the level of the attached muscles, and laid outer surface uppermost on the paper of the machine. It was cut to strips 0·35 or 0·2 mm. in thickness. These tended to collect on the blade rather than to remain on the paper.

### *Metabolic measurements*

The metabolic characteristics which are dependent on cell structure and which it was desired to preserve in the chopped tissue, are exhibited by sliced, but not by 'homogenized', tissues. In the following studies, the chopped preparations were therefore compared with 'homogenates' and, especially, with slices from the same material. Some data regarding the relative size and area of the preparations used are given in Table 1.

Table 1. *Weight and area of different tissue preparations*

(Tissue density is taken as 1. 70 mg. is the weight of tissue used in typical experiments, suspended in 3·5 ml. fluid.)

| Preparation (mm.) | No. of pieces in a specimen of wt. 70 mg. | Wt. of individual fragments (mg.) | Area of individual fragments (sq.mm.) | Area of pieces in 70 mg. sample (sq.mm.) |
|---|---|---|---|---|
| Slice 0·35 × 20 × 10 | 1 | 70 | 421 | 421 |
| Slice fragments 0·35 × 2·5 × 4 | 20 | 3·5 | 24·6 | 491 |
| Chopped as prisms 0·2 × 0·2 × 2 | 875 | 0·08 | 1·68 | 1470 |
| Chopped as prisms 0·067 × 0·067 × 2 | 7875 | 0·009 | 0·545 | 4292 |

Table 2. *Level and persistence of respiration in preparations of guinea pig cerebral cortex*

(The tissue was cut or 'homogenized' about 3 min. after death of the animals, and respiratory measurements began 25 min. after death, using glucose-phosphate saline. The 'homogenate' was made in a glass apparatus (Potter & Elvehjem, 1936) with 1 part of tissue and 3 parts of the same saline, 'homogenizing' for 10 sec. Slices were 0·35 mm. thick and cut by hand with a blade and template. Chopped tissue was prepared with the machine set to give prisms 0·21 × 0·21 mm. square in section.)

| Preparation | Respiration ($\mu$moles $O_2$/g./hr.) at different times after placing in thermostat | | | |
|---|---|---|---|---|
| | 0–30 min. | 30–60 min. | 60–90 min. | 100–130 min. |
| Sliced 18–22 mg./ml. | 63, 57, 61 | — | 63, 59, 60 | 65, 62, 60 |
| Chopped 18–22 mg./ml. | 55, 61, 59 | — | 57, 62, 57 | 57, 59, 56 |
| 'Homogenized' 83 mg./ml. | 33 | 27 | 24 | — |
| 'Homogenized' 42 mg./ml. | 23 | 19 | 24 | — |
| 'Homogenized' 21 mg./ml. | 24 | 18 | 13 | — |

*Respiration and aerobic glycolysis of preparations from cerebral cortex.* Measurements were made with glucose as substrate in a balanced saline which supported respiratory rates of 60–75 $\mu$moles/g./hr. for slices of guinea pig or rat cortex. Table 2 and the first column of Table 3 show that tissue chopped in the machine respired at rates comparable to those of slices made in the ordinary fashion. Sufficient data are available in Tables 2 and 3 to suggest that the chopped tissue has rates of respiration only some 1–3 % lower than that of slices. 'Homogenized' tissue, on the other hand, gave initial values some 60 % lower than slices when examined at comparable tissue concentrations. Much higher rates of respiration can be obtained for 'homogenates' of cerebral tissues when these are examined at higher concentrations and in enriched media (see, for example, Elliott & Libet, 1942; Case & McIlwain, 1951), but such conditions are not relevant to the present comparison. Moreover, the respiration of chopped tissue was practically as stable with time as was that of the slices normally used in metabolic studies (Table 2). Whereas respiration of 'homogenates' had fallen to 50–60 % of its original rate in 90 min., that of the chopped tissue was stable for periods of over 2 hr.

The lactic acid which accumulated during aerobic metabolism with glucose as substrate was also determined (Table 3). The accumulation in experiments with machine-chopped tissue and with hand-cut slices was similar, but significantly greater with the chopped tissue. Such accumulation is a relatively labile property of the tissue, and changes considerably during the progress of an experiment with sliced tissue (McIlwain & Grinyer, 1950). The difference between the accumulation with chopped and sliced tissue is less than that which occurred spontaneously with time, during experiments with slices.

In a few instances, human cerebral tissues were available in sufficient quantity for slices to be compared with chopped preparations (Table 3). The respiratory rates were in all cases lower than those with rat or guinea pig (cf. Elliott & Penfield, 1948; McIlwain *et al.* 1952). Comparison between sliced and chopped tissue gave results similar in all respects to those with animal tissues.

*Metabolic response to electrical stimulation*

Little response to electrical stimulation is shown in simple 'homogenates' in the present medium (McIlwain, 1951*a*) in distinction to the effect clearly shown by slices. Mechanically chopped tissue behaved in this respect like slices (Tables 2 and 3). Respiration of both preparations of guinea pig cerebral cortex nearly doubled with the greatest stimulus. With the lesser stimulus, response of the chopped preparation was significantly lower than the response of slices. With preparations from rat, the same tendencies were evident. The respiration of human cerebral cortex, after chopping, also responded well to stimulation.

The aerobic formation of lactic acid by cerebral tissues also increased on application of electrical

Table 3. *Metabolic response to electrical impulses in mechanically chopped and in hand-sliced cerebral cortex*

(Experiments were in vessels *E* (McIlwain, 1951*b*) with concentric electrodes between which the tissue floated. About 70 mg. of tissue were used in each case, with 3·5 ml. of glucose-phosphate saline. The slices always consisted of approx. 20 pieces (thickness 0·35 mm.) in order that they, like the chopped tissue, should be distributed between the electrodes. Respiration was determined from 5 min. manometric readings. The values which are summarized in column *a* were derived from vessels observed for periods of at least 30 min., and in columns *b* and *c* are derived from subsequent periods of observations with some of the same vessels (not every vessel being subjected to the two voltage ranges). Accumulation of lactic acid was determined by Barker & Summerson's (1941) method in the vessel contents at the end of the manometric experiments. The values of column *d* are derived from vessels not exposed to impulses, and of column *e* from vessels which were so exposed and of which the respiration furnished data incorporated in columns *b* and *c*.

With a given species, the individual experiments from which are derived the values for tissues 'sliced' and 'chopped at 0·21 mm.' were matched with respect to length of metabolism and impulse characteristics so that the mean values for the groups are comparable. The length of experiments and impulse characteristics varied between different experiments in the same group, and this contributed to the variation in values for lactic acid (see McIlwain, Anguiano & Cheshire, 1951).

Under 'respiration' and 'accumulation of lactic acid', mean values are in bold type, with below, the number of individual values and S.D. Individual values marked by an asterisk (*).)

| | | Respiration | | | Accumulation of lactic acid | | |
|---|---|---|---|---|---|---|---|
| | | *a* | *b* | *c* | *d* | *e* | *f* |
| Species | Preparation | Unstimulated ($\mu$moles $O_2$/ g./hr.) | Stimulated at 10–12 V. (% of *a*) | Stimulated at 18–22 V. (% of *a*) | Unstimulated ($\mu$moles/ g./hr.) | Increase ($\mu$moles/ g./hr.) | Stimulated rate (% of *d*) |
| Rat | Sliced by hand | **75** (12; 4·0) | **145** (3; 4·9) | **180** (6; 18) | **31** (4; 12·3) | **48** (5; 17·9) | **274** (5; 54) |
| Rat | Chopped at 0·21 × 0·21 mm. by machine | **72·3** (12; 6·9) | **131** (3; 8·5) | **168** (6; 16) | **35** (4; 12·5) | **31** (5; 9·2) | **202** (5; 35) |
| Rat | Chopped at 0·1 × 0·1 mm. by machine | **56** (4; 5·3) | **139** (4; 14·1) | **181** (4; 15) | 24*, 28* | 56*, 38* | 332*, 236* |
| Guinea pig | Sliced by hand | **60·8** (18; 6·0) | **157** (8; 15) | **193** (7; 25) | **21** (7; 5) | **39** (9; 12·3) | **288** (9; 51) |
| Guinea pig | Chopped at 0·21 × 0·21 mm. by machine | **59·7** (18; 5·5) | **133** (8; 14) | **192** (7; 22) | **35** (7; 5·6) | **37** (9; 17·8) | **203** (9; 43) |
| Guinea pig | Chopped at 0·067 × 0·067 mm. by machine | **57·0** (4; 8·9) | 117* | 153* | 21*, 26* | 16*, 28* | 176*, 218* |
| Man | Sliced by hand | **36·0** (6; 4·0) | 126*, 149* | 197*, 188* | 33* | 25* | 176* |
| Man | Chopped at 0·21 × 0·21 mm. by machine | **38·7** (6; 6·0) | 166*, 129* | 206*, 188* | 44* | 41* | 193* |

impulses (Table 3). The increase was large, and corresponded to a doubling or trebling of the rate of accumulation of the acid during the periods of stimulation. The increase with chopped tissue approached that with sliced tissue, when measured by the increase in lactic acid formed. When the rate of formation of lactic acid during stimulation was expressed as a percentage of its unstimulated rate of formation, values for the chopped tissue were, however, smaller. The more rapid formation of lactic acid in unstimulated chopped tissue contributed to this.

*Effect of fineness of chopping and direction of cut.* A few experiments are included in Table 3, in which tissue has been chopped to prisms of cross-section 0·1 × 0·1 mm. and 0·067 × 0·067 mm. Both respiration and aerobic accumulation of lactic acid were somewhat lower than with tissue chopped to pieces of cross-section 0·21 × 0·21 mm. The response to electrical stimulation was not markedly altered in the preparation at 0·1 × 0·1 mm., but was less in the instance examined at 0·067 × 0·067 mm. The individual fragments have then one-quarter or one-ninth of the volume of fragments of the usual size; for other data, see Table 1.

Tissues have normally been chopped in two directions at right angles to each other, and each at right angles to the outer surface of the tissue specimen. With cerebral cortex from experimental animals this was also at right angles to the lateral ventricles. In biopsy specimens such a surface is not always available, and the effect of direction of cut was therefore examined. The usual block was cut with a scalpel to strips about 2 mm. in width, these were turned on their main axis by 90°, and then chopped in the usual manner. Their respiration and its response to electrical stimulation were normal.

*Other metabolic observations.* The following findings are the results of one or two experiments in each case, and metabolic rates are not quoted. The respiration rates of mechanically chopped (0·21 × 0·21 mm.) and hand-sliced cerebral cortex were compared in

glycylglycine-glucose saline, in the absence and presence of electrical impulses, without finding marked differences between the preparations. The approximate course of aerobic acid formation from glucose in the bicarbonate saline was followed with the same result. The tissue chopped at 0·21 × 0·21 mm. should be fine enough to be adequately oxygenated by air, and rates of respiration were found to be very similar in air and oxygen.

## DISCUSSION

Most of the apparatus introduced during the past 20 years for preparing tissues for biochemical work destroys cell structure. This is true of Potter & Elvehjem's (1936) homogenizer, of the Waring Blendor, and of other similar apparatus (e.g. that of Folley & Watson (1948)). A notable exception is Stadie & Riggs's (1944) template for cutting slices (see also Deutsch & Raper, 1936), but this requires larger specimens of tissue than normal slicing techniques and so was not applicable to the present problems.

From time to time, experimental descriptions have included references to tissue preparations largely made manually, but which were probably partly analogous to the present one, without, however, giving full descrptions of the methods or of the properties of the product in comparison with other methods of preparing tissues. Some (but not all) minces, breis, and scissor-cut tissues may have approached the condition here described as chopped. Dickens (1941) briefly reviewed such methods and pointed out that they were then much less used than before. The articles edited by Potter (1948), which include descriptions of a variety of methods of preparing tissues, do not describe a method comparable to chopping. It would appear that the introduction of blenders and homogenizers had largely displaced the manual preparation of breis. This is understandable as Potter & Elvehjem's (1936) apparatus is very convenient, is applicable to small specimens, and was immediately applied with success to problems which required breakdown of cell structure. That it was also applied to problems which would better have been tackled with preparations retaining cell structure, was largely overlooked.

The relatively indefinite nature of manually prepared 'breis' and analogous preparations also contributed to their replacement, though it was later realized that 'homogenates' also vary considerably in biochemical properties according to whether they are 'fine' or 'coarse'—properties usually not adjusted quantitatively but assessed according to an approximate estimate of the degree of fit of the homogenizer, the force exerted during its use or the length of time for which it was run. By contrast, fineness of cut in the present machine is predetermined and reasonably precise. Moreover, it can be made in planes selected in relation to structural elements in the tissue being cut, and in many cases yields suspensions which can be pipetted and the tissue distributed by volume of suspending fluid. Blenders are akin to the present machine in depending on speed of the cutter and inertia of the tissue for their disintegrating effect, which is, however, random and very destructive of cells.

The tables indicate that fragments of cerebral cortex of only a few micrograms in weight behave metabolically in a fashion radically different from 'homogenates' of the same tissue. It is especially noteworthy that metabolic response to electrical impulses is retained in such minute fragments. In general, machines of this type would appear to have a wide range of potential uses, much beyond those for which the present model was designed.

## SUMMARY

1. A machine is described which produces slices and suspensions of fresh tissues with minimum disruption of their cellular structure. It reduced specimens of several animal tissues to slices down to 0·2 mm. in thickness, and to prisms down to 0·067 × 0·067 mm. in cross-section. It has been applied to liver, spleen, kidney, adrenal, lung, heart, abdominal wall, skin, and especially to tissues of the central nervous system.

2. A convenient preparation which can be made from many tissues in 30 sec. consists of prisms 0·2 × 0·2 mm. in section and 2 mm. long. This can be suspended in salines, and sampled as a suspension.

3. Many such suspensions were made from rat, guinea pig, and human cerebral hemispheres, and compared metabolically with slices or 'homogenates' from the same tissues. In a glucose saline suitable for respiration of tissues, the chopped material respired at rates within 3% of those of ordinary hand-cut slices, and markedly more rapidly than 'homogenates'. Respiration by the mechanically chopped tissue was stable with time over a period of at least 2 hr., during which that of 'homogenates' fell considerably. Aerobic accumulation of lactic acid in systems with the chopped tissue was similar to, but in some cases rather greater than, that in systems with ordinary slices.

4. The rates at which mechanically chopped cerebral cortex respired and formed lactic acid were doubled or trebled by the electrical impulses which induced such changes in sliced cerebral cortex.

5. The properties described in paragraphs 3 and 4 indicate that structure which is lost in 'homogenates' is largely retained in the chopped pre-

parations. The present method is especially applicable to preparing biopsy and other small specimens of tissue for metabolic examination of processes which are controlled by structural factors.

We are greatly indebted to Mr W. Piper for taking part in the construction of the machine, to Mrs O. Forda for assisting in some of the metabolic experiments, and to Mr S. Jaffe for the photographs.

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## EXPLANATION OF PLATES 1 AND 2

### PLATE 1

Figs. 1 and 2. The chopper. *A*, cutting-arm carrying blade *B*; *H* 1, handwheel on shaft *Sh* 1 which also carries the two cams; *H* 2, handwheel on shaft *Sh* 2 which also carries the leadscrew. *T*, cutting table on the carriage *C* which is moved by the leadscrew. *Bl*, adjustable block whose position in the frame *F* conditions the thickness of section cut.

### PLATE 2

Guinea pig tissues prepared with the chopper and photographed, while indirectly illuminated, in a shallow layer of saline in dishes with black background. The whole of the specimens chopped have been transferred for photographing.

Fig. 4. Cerebellum cut at 0·26 mm. intervals. Note the cortical layers and white matter which show little tendency to separate. There is also little debris.

Fig. 5. Cerebral hemisphere cut at 0·21 mm. intervals in two directions at right angles. The specimen cut was a block 5 mm. square taken from about the middle of the hemisphere, extending from its cortical surface to the lateral ventricle. For cutting, it was laid on the subcortical white matter. A portion of this is seen at one end of each fragment.

Fig. 6. Liver cut at 0·3 mm. intervals in two directions at right angles.

Fig. 7. Kidney cut at 0·26 mm. intervals.

# The Occurrence of *m*-Hydroxybenzoic Acid in Urine

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(*Received* 5 *August* 1952)

In a study of the excretion of oxidation products of naphthalene by rabbits (Boyland & Wiltshire, 1952), 1-naphthol was estimated in urine as the blue indophenol formed with 2:6-dichloroquinone chloroimide (phenol reagent). Under the conditions described at pH 9·2, this indophenol was completely extracted into butanol. It was noticed that the urine of rats and rabbits dosed with naphthalene or 1-naphthol contained in addition a phenol which reacted to give a blue indophenol which was not extracted by butanol. The compound could not be detected regularly in urine of normal animals by this method, but it could be detected and estimated by chromatography and was isolated from such urine. The amount of the substance appeared to be increased by acid hydrolysis. The compound isolated was shown to be *m*-hydroxybenzoic acid, which Bray, Lake, Thorpe & White (1950) had detected by paper chromatography as a constituent of normal rabbit urine.

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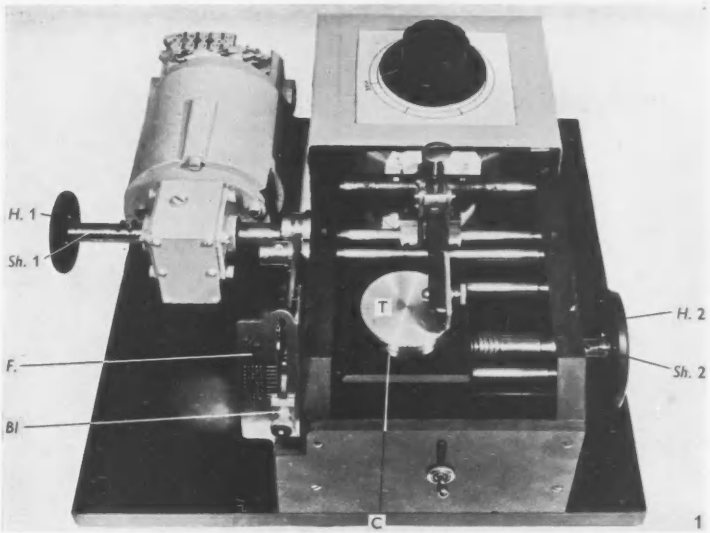


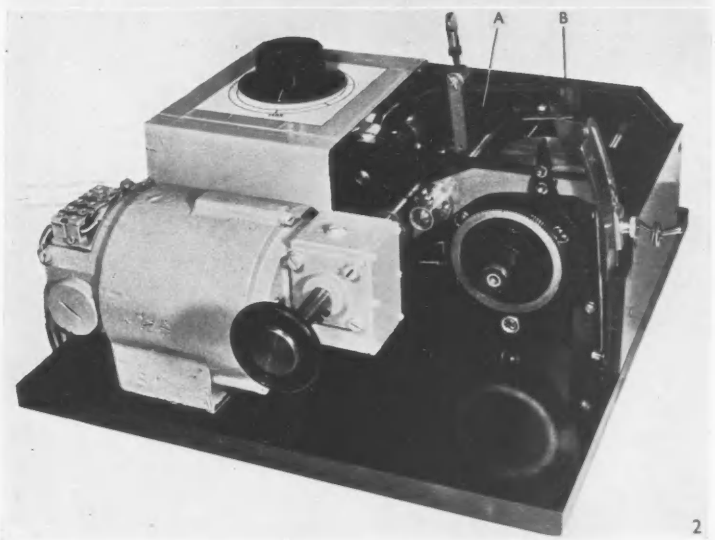


Figs. 1–2.

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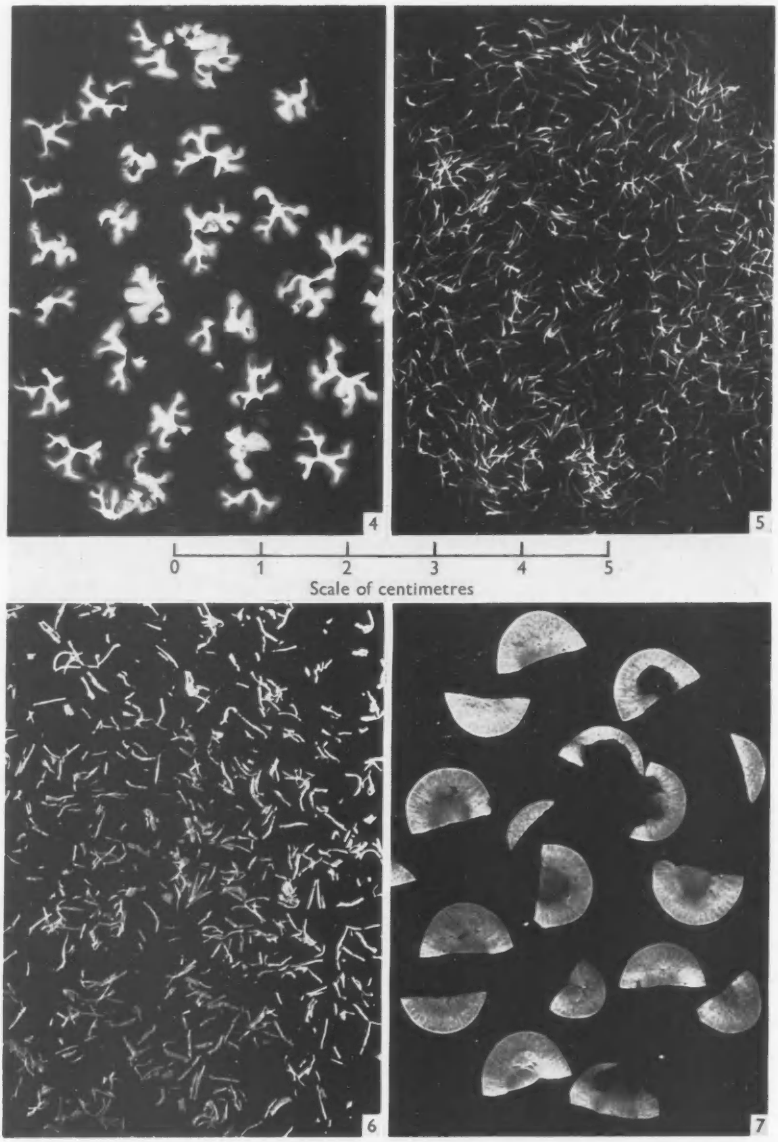


Figs. 4–7.

## EXPERIMENTAL

*Reaction of phenols with the phenol reagent.* A number of compounds which react with 2:6-dichloroquinone chloroimide has been listed by Fearon (1944). Extraction of the colour with butanol was used in the present work to avoid interference from coloured products in urine. The extraction also separated phenols into four classes (Table 1) as follows:

(1) Monophenols substituted in the position para to the phenolic hydroxyl gave a green, purple or yellow colour or no reaction, in the butanol.

(2) Other para-substituted phenols containing a second hydroxyl or an acidic group gave similar colours in the alkaline buffer or no reaction.

(3) Monophenols not substituted in the para position gave a blue colour in butanol.

(4) Other phenols with a free para position, but with a second hydroxyl or an acidic group, gave a blue, violet or magenta colour in the alkaline buffer.

Anomalous reactions were observed with naphthoresorcinol and 3-hydroxyphthalic acid (Table 1). All specimens of urine from rabbits dosed with naphthalene or 1-naphthol and some specimens of urine from undosed rabbits which were tested gave a blue colour which was not extracted with butanol. This reaction was presumably due to the presence of phenol with no substitutent in the position para to the hydroxyl group and a second acidic group (e.g. carboxyl or phenolic hydroxyl).

*Isolation of* m*-hydroxybenzoic acid from urine of rabbits dosed with naphthalene.* (All melting points are uncorrected.) Five rabbits were dosed daily, each with 0·25 g. naphthalene, and the collected urine centrifuged to remove insoluble material. It was acidified to pH 2 by addition of HCl and warmed to 90°. When effervescence had ceased it was cooled and extracted three times with 0·2 vol. ether. Colorimetric tests with the phenol reagent showed that transfer of the required substance was practically complete. The ether extract was washed repeatedly with water and the acidic compounds extracted with 2% $NaHCO_3$ solution. The $NaHCO_3$ extract was washed with ether, concentrated to 100 ml. and acidified to pH 4 with HCl. On standing at 0° for 12 hr., crystalline material separated which did not

Table 1. *Colour reactions of phenols with* 2:6-*dichloroquinone chloroimide at* pH 9·2

(The colour was developed in 0·1 M-$Na_2B_4O_7$ and extracted with an equal volume of *n*-butanol.)

| | Colour in $Na_2B_4O_7$ | Colour in butanol |
|---|---|---|
| Monophenols | | |
| (*a*) Substituted in para position | | |
| *p*-Cresol | None | None |
| 2-Naphthol | None | Green |
| Di-2-naphthol | None | Purple |
| (*b*) Not substituted in para position | | |
| Phenol | None | Blue |
| 1-Naphthol | None | Blue |
| 2-Nitroso-1-naphthol | None | Green |
| Polyphenols | | |
| (*a*) Substituted in para position | | |
| Quinol | Yellow | Yellow |
| (*b*) Not substituted in para position | | |
| Catechol | Violet | Blue |
| Resorcinol | Magenta | Blue |
| Orcinol | Magenta | Blue |
| Resorcyl aldehyde | None | Blue-green |
| Naphthoresorcinol | Orange | None |
| Monophenols with acidic group | | |
| (*a*) Substituted in para position | | |
| *p*-Hydroxybenzoic acid | None | None |
| Sulphosalicyclic acid | None | None |
| 2-Hydroxy-1-naphthoic acid | Green | Purple |
| (*b*) Not substituted in para position | | |
| Salicyclic acid | Blue | None |
| *m*-Hydroxybenzoic acid | Blue | None |
| 3-Hydroxyphthalic acid | None | None |
| Polyphenols with acidic group | | |
| (*a*) Substituted in para position | | |
| Gentisic acid | None | None |
| Homogentisic acid | None | None |
| (*b*) Not substituted in para position | | |
| γ-Resorcylic acid | Magenta | None |
| 3:4-Dihydroxyphenylalanine | Pink | Violet |

react with phenol reagent. This deposit was purified and found to contain naphthalene mercapturic acid (m.p. 169°) and benzoic acid (m.p. 122°). The filtrate was further acidified to pH 2 and extracted with benzene. This removed the remaining benzoic acid, but very little of the phenolic acid. The aqueous solution was concentrated to 5 ml. and filtered while hot. On cooling, there were deposited colourless needles, which gave the appropriate reaction with phenol reagent. These were recrystallized once from hot water and once from benzene, m.p. (alone and mixed with an authentic sample of *m*-hydroxybenzoic acid) 199–200°. Neutralization equivalent found, 148; calc. for hydroxybenzoic acid, 138. Analysis (Found: C, 60·6; H, 4·3. Calc. for $C_7H_6O_3$: C, 60·9; H, 4·4%). The hydroxy acid (0·02 g.) set aside in pyridine with acetyl chloride for 12 hr. gave the *O*-acetyl derivative m.p. (alone and with authentic *m*-acetoxybenzoic acid) 130–131° after crystallization from xylene. (Found: C, 60·0; H, 4·7. Calc. for $C_9H_8O_4$: C, 60·0; H, 4·5%.)

Table 2. *Amounts of* 3*-hydroxybenzoic acid isolated from urine*

| Source of urine | Volume of urine (l.) | Amount isolated (mg.) | Amount (mg./l.) |
|---|---|---|---|
| Five rabbits each of which received 0·25 g. naphthalene daily for 3 consecutive days | 2 | 31 | 15 |
| Five rabbits each of which received 0·25 g. naphthalene daily for 3 consecutive days | 2 | 58 | 29 |
| Five normal rabbits | 3 | 19 | 6 |
| Five normal rabbits | 4·7 | Nil | — |
| Five normal rabbits | 10 | 29 | 3 |

*Isolation of* m*-hydroxybenzoic acid from urine of normal rabbits.* Urine of normal rabbits was treated as described for the urine of rabbits dosed with naphthalene. Concentration of the acidified $NaHCO_3$ extract gave no crystalline material but only a black tar. This was extracted with 50% $CHCl_3$: 50% methanol. (Preliminary trial showed that *m*-hydroxybenzoic acid could be adsorbed on alumina and recovered in 90% yield by elution with $NH_3$.) The extract of the tar was added drop by drop to an alumina column (Savory & Moore) (3 × 6 cm.), prepared in benzene. After adsorption the column was eluted with the following solvents: $CHCl_3$; 50% $CHCl_3$/50% methanol; 25% $CHCl_3$/75% methanol; methanol and finally 300 ml. $NH_3$, sp.gr. 0·88. The ammoniacal eluate was acidified and extracted with ether. The ether was evaporated to yield a gummy residue which was sublimed at 150° (0·2 mm. Hg). The sublimate crystallized from benzene to give *m*-hydroxybenzoic acid, m.p. (alone and with an authentic specimen) 197–198°.

The yields of acid isolated are shown in Table 2.

Acetylation of the product isolated from urine of undosed rabbits with acetyl chloride in pyridine gave the acetate, m.p. (alone and mixed with an authentic specimen) 129–130°. (Found: C, 60·0; H, 4·9. Calc. for $C_9H_8O_4$: C, 60·0; H, 4·5%.)

*Properties of the compound.* The compound gave a blue colour with the 2:6-dichloroquinone chloroimide phenol reagent and the colour was not extracted by butanol. It gave no colour with $FeCl_3$ solution (salicyclic acid gave a characteristic violet colour) and a red colour with diazotized *p*-nitroaniline. It did not reduce ammoniacal $AgNO_3$ solution. It was more soluble in hot water than in cold, very soluble in methanol, ethanol, butanol and ether, but only slightly soluble in benzene and light petroleum.

*Separation and estimation of* m*-hydroxybenzoic acid by paper partition chromatography.* Urinary phenolic acids were separated by ascending development on sheets of Whatman no. 1 (special chromatographic) paper hung in a tank so that the bottom of the paper was in contact with butanol saturated with 0·005 N-$NH_4OH$. After development for approximately 40 hr. at room temperature the papers were removed and dried at room temperature. The phenolic acids were detected by spraying successively with freshly diazotized 0·0005 M-*p*-nitroaniline solution and then with M-$Na_2CO_3$, when the *m*- and *p*-hydroxybenzoic acids were revealed as red spots.

Typical $R_F$ values under these conditions were: *o*-hydroxybenzoic acid 0·46, *p*-hydroxybenzoic acid 0·33, *m*-hydroxybenzoic acid 0·26, *m*-hydroxyhippuric acid 0·22 and *p*-hydroxyhippuric acid 0·19.

Analyses for free *m*-hydroxybenzoic acid were carried out on ether extracts obtained by continuous extraction of the urines at pH 2 for 20 hr. Ether-insoluble *m*-hydroxybenzoic acid conjugates were hydrolysed by adjusting the acid content of the extracted urine to 2 N with $H_2SO_4$ and boiling under reflux for 1 hr. The hydrolysed urines were adjusted to pH 6 and continuously extracted with ether for 20 hr. The ether extract was then evaporated to dryness and the residue dissolved in 40% (v/v) aqueous ethanol (1–5 ml.).

Quantitative estimations of *m*-hydroxybenzoic acid were made using a relationship between spot area and the

Table 3. *The* m*-hydroxybenzoic acid content of some animal and human urine estimated by paper chromatography*

| | Free *m*-hydroxybenzoic acid | | Conjugated *m*-hydroxybenzoic acid | | Total *m*-hydroxybenzoic acid |
|---|---|---|---|---|---|
| | mg./100 ml. urine | Daily excretion (mg.) | mg./100 ml. urine | Daily excretion (mg.) | Total daily excretion (mg.) |
| Rabbits (F.) | 6·9 | 15 | 4·7 | 10 | 25 |
| Rabbits (F.) | 7·0 | 15 | 4·9 | 10 | 25 |
| Rabbits dosed with naphthalene | 4·7 | 10 | 10·1 | 20 | 30 |
| Rats (F.) | Nil | Nil | Nil | Nil | Nil |
| Humans (M.) | 0·6 | 8·8 | 0·08 | 1·2 | 10·0 |
| Humans (F.) | 1·0 | 15·0 | 0·04 | 0·6 | 15·6 |

logarithm of its acid content (Fisher, Parsons & Holmes, 1949). Two standard solutions of *m*-hydroxybenzoic acid and two dilutions of an ether extract were applied to the paper serially by Exelo pipettes (5 $\mu$l.). Five applications of each solution were made on each sheet of paper, two sheets of paper being used for each determination. After chromatographic development in the same tank and detection of the acid with diazotized *p*-nitroaniline reagent the papers were dried at room temperature. The spots of the *m*-hydroxybenzoic acid dye were traced on graph paper (ruled in 1 mm. squares) from which accurate estimations of areas were obtained. From the mean area the concentration of *m*-hydroxybenzoic acid in a urine extract was found from the formula used by Fisher *et al.* (1949) with errors of $\pm$2–7%.

The *m*-hydroxybenzoic acid content of female rats and rabbits and adult male and female human urines have been determined by this method (Table 3). No *m*-hydroxyhippuric acid could be detected in any of the rat urines examined.

## DISCUSSION

The isolation of *m*-hydroxybenzoic acid has been reported from the urine of rabbits dosed with $\beta$-ionone (Bielig & Hayasida, 1940), urine of pregnant mares (Lederer & Polonsky, 1948) and the scent gland of the beaver (Lederer, 1941, 1949) and from the chemical degradation of juglone (Bernthsen & Semper, 1886). Fischer & Bielig (1940) found that rabbits fed with a number of unsaturated aromatic compounds excreted *m*-hydroxybenzoic acid (which was not present in normal rabbit urine examined by them) and that there was a concurrent increase in the excretion of benzoic acid.

Quick (1932), Williams (1938) and Bray, Ryman & Thorpe (1948) investigated the fate of *m*-hydroxybenzoic acid administered to animals. In men, *m*-hydroxyhippuric acid is the main product; in the dog, a glucuronide. In rabbits 70–97% is excreted as free acid, 6–11% as ester glucuronide, 2–19% as ether glucuronide and 2–4% as ethereal sulphate. Gentisic acid may also be formed.

It was suggested by Fischer & Bielig (1940) that hydroxybenzoic acids might be formed by oxidation of benzoic acid. However, Smith & Williams (1950) were unable to detect any of the three monohydroxybenzoic acids after dosing rabbits with benzoic acid. Lederer (1949) mentioned *m*-tyrosine and *m*-hydroxyanthranilic acid as possible precursors. The latter compound is known to be converted to quinolinic acid in liver (Henderson & Ramasarma, 1949), and it was suggested it might be deaminated by intestinal bacteria. *m*-Tyrosine has not so far been found in animals.

The isolation of the *m*-hydroxybenzoic acid from urine of rabbits which were dosed with naphthalene and from urine of undosed rabbits indicates that it may be the metabolic product of some food constituent as yet unidentified. The acid was isolated in a pure form more easily from the urine of naphthalene-treated rabbits than from untreated rabbits. It was also detected more easily in naphthalene urine by the 2:6-dichloroquinone chloroimide reagent, but the estimation by the paper-chromatographic method indicated no difference in the concentration of the acid in the two types of urine.

## SUMMARY

*m*-Hydroxybenzoic acid was estimated in and isolated from urine of rabbits injected with naphthalene and from untreated rabbits.

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# Metabolism of Naphthalene by Liver Slices

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The metabolism of naphthalene in animals was reviewed by Young (1950). Since that review was published experiments have shown that the oxidative pathways leading to 1-naphthol and to 1:2-dihydroxy-1:2-dihydronaphthalene (naphthalenediol), are probably separate and independent (cf. Boyland & Wiltshire, 1952). The conversion of naphthalene to naphthalene mercapturic acid (Bourne & Young, 1934) probably involves still another metabolic route. The present experiments indicate that isolated slices of rat liver are able to oxidize naphthalene to naphthalenediol.

## EXPERIMENTAL

(*a*) Tissue slices not more than 0·5 mm. thick were weighed in a moist condition on a torsion balance, and 100 mg. quantities placed in Krebs-Ringer phosphate solution in Warburg vessels with 0·2 ml. 2N-KOH in the inner vessel. Naphthalene and other additions were made as shown in Table 1, so that the total volume was 3·4 ml. The vessels were equilibrated with $O_2$ and, at the end of the incubation (usually 2 hr.), the KOH was removed and *n*-butanol (5 ml.) was added to the incubated mixture. The flasks were shaken mechanically for 30 min. and the mixtures centrifuged. Portions (4 ml.) of the butanol

Table 1. *Formation of substance (probably* 1:2-*dihydroxy*-1:2-*dihydronaphthalene) which yields* 1-*naphthol in* N-HCl *in* 15 *min. at* 100° *by rat-liver slices*

(Reaction vessels contained 100 mg. wet tissue in 3·4 ml. Ringer-phosphate pH 7·4. Temperature 37°. Gas phase $O_2$.)

| Rat no. | Naphthalene present (μg./ml.) | Condition of rat | Additions to system | $Q_{O_2}$ | Apparent 1:2-dihydroxy-1:2-dihydronaphthalene (μg. 1-naphthol formed/g. tissue/hr.) |
|---|---|---|---|---|---|
| 1 | 30 | Normal | None | 3·1 | 50 |
| 2 | 30 | Fed on 40% protein diet | None | 3·0 | 40 |
| 3 | 30 | Normal | None | 3·2 | 25 |
| | 30 | Normal | 0·25% Tetramethyluric acid | 3·25 | 85 |
| 4 | 30 | Fed on 40% protein diet | None | 7·2 | 50 |
| | 30 | Fed on 40% protein diet | 0·25% Tetramethyluric acid | 6·4 | 60 |
| 5 | 30 | Fed on 40% protein diet | None | 3·6 | 85 |
| | 30 | Fed on 40% protein diet | 0·05% Tetramethyluric acid | 5·2 | 75 |
| | 30 | Fed on 40% protein diet | 0·1% Tetramethyluric acid | 6·2 | 60 |
| | 30 | Fed on 40% protein diet | 0·1% Glucose | 4·2 | 75 |
| | 30 | Fed on 40% protein diet | 0·1% Glucose + 0·05% tetramethyluric acid | 3·5 | 75 |
| | 30 | Fed on 40% protein diet | 0·1% Glucose + 0·1% tetramethyluric acid | 4·2 | 120 |
| 6 | 30 | Fed on 20% protein diet | 0·1% Glucose | 5·2 | 40 |
| | 30 | Fed on 20% protein diet | 0·1% Glucose + 0·1% tetramethyluric acid | 4·9 | 60 |
| | 30 | Fed on 20% protein diet | 0·1% Glucose + 0·1% nicotinamide | 3·8 | 30 |
| | 30 | Fed on 20% protein diet | 0·1% Glucose + 0·2% nicotinamide | 3·6 | 30 |
| | 30 | Fed on 20% protein diet | 0·1% Glucose + 0·1% tetramethyluric acid + 0·01% ascorbic acid | 4·1 | 90 |
| 7 | 0 | Normal | 0·1% Tetramethyluric acid | 4·8 | 0 |
| | 10 | Normal | 0·1% Tetramethyluric acid | 3·5 | 0 |
| | 20 | Normal | 0·1% Tetramethyluric acid | 4·4 | 12 |
| | 30 | Normal | 0·1% Tetramethyluric acid | 5·5 | 35 |
| | 80 | Normal | 0·1% Tetramethyluric acid | 4·5 | 80 |
| | 200 | Normal | 0·1% Tetramethyluric acid | 3·9 | 110 |

extracts were removed and heated with 0·2 ml. 10 N-HCl at 100° for 10 min. (This procedure was shown to convert synthetic DL-1:2-dihydroxy-1:2-dihydronaphthalene quantitatively into 1-naphthol.) The butanol was cooled and 1 ml. 0·01 M diazotized sulphanilic acid added, followed by 1 ml. 4 N-NaOH and 4 ml. ethanol. The red colour of the solution was measured in a photoelectric colorimeter using a green filter. Standard solutions of naphthalenediol and of 1-naphthol were treated in the same way for comparison.

(*b*) Tissue slices (500 mg. wet wt.) were suspended in 10 ml. Krebs-Ringer phosphate solution at pH 7·4 and incubated at 37° in 50 ml. conical flasks. Naphthalene was added as a 10% solution in methyl cellosolve. The flasks were shaken slowly and gassed with a stream of $O_2$. At the end of the incubation period the slices were removed and the reaction mixture was transferred to 50 ml. centrifuge tubes, the flasks being rinsed into the centrifuge tubes with 20 ml. ethanol. The tubes were warmed to 60° and the coagulated protein centrifuged down.

Naphthol was estimated colorimetrically in 1 ml. portions of the supernatant fluid acidified with 0·1 ml. 10 N-HCl before and after heating for 15 min. at 100°, by reaction with 2:6-dichloroquinone chloroimide as described by Gibbs (1927). The solution was neutralized by dropwise addition of saturated $Na_2CO_3$ and 1 ml. 0·1 M-$Na_2B_4O_7.10H_2O$, and shaken with 10 ml. *n*-butanol and 0·2 ml. ethanolic 2:6-dichloroquinone chloroimide. After standing for 30 min. the upper layer was centrifuged and read against a blank in a Spekker colorimeter using filter OY2. The naphthol estimated in the unheated aliquot represents the free 1-naphthol while that in the heated solution would contain free 1-naphthol plus naphthol derived from 1-naphthylsulphuric acid and 1:2-dihydroxy-1:2-dihydronaphthalene.

In two experiments ethanol was removed from the supernatant, and the remaining aqueous solution neutralized to pH 7·4 and extracted twice with an equal volume of ether. Subsequent analysis of the aqueous and ethereal layers gave separate estimates of the 15 min. naphthol due to naphthylsulphuric acid and naphthalenediol.

(*c*) Rat-liver homogenates were prepared in a glass homogenizer cooled in ice. Rabbit-liver and kidney homogenates were made in a Waring Blendor in a cold room at +2°. The tissue was removed immediately after killing the animal, weighed and cooled on ice. It was then chopped with scissors and ground for 30 sec. in 2 vol. of ice-cold 0·15 M-KCl solution. Other homogenates of rat-kidney and liver were made in glass homogenizers using water or 0·2% sodium citrate.

## RESULTS

(1) Some of the experimental results obtained by the technique described in the experimental method under (*a*) are given in Table 1. Preliminary experiments showed that incubation of 0·01 mg. 1:2-dihydroxy-1:2-dihydronaphthalene with 100 mg. of liver slices or with 1 ml. of 1:10 liver homogenate for 1 or 2 hr. caused no decrease in the diol as estimated by the method. The results of the last experiment (rat 7) suggest that the amounts of diol formed vary with the initial amount of naphthalene present, although in this experiment never more than 4% of the naphthalene present was transformed into diol and the highest yield of diol (120 μg./g. tissue/hr.) represents only 10% conversion of the naphthalene added to the medium. The comparison of the rate of reaction in rats fed on a normal (approx. 20% protein diet) and a 40% protein diet indicate that the maintenance of the animals on the higher protein diet for 3–5 days before using the liver did not affect the ability of the liver to oxidize naphthalene. The oxidation was not increased by addition of glucose, but was generally increased by addition of tetramethyluric acid (which forms a water-soluble complex with naphthalene).

(2) Data obtained by the second technique (*b*) are given in Table 2. These results show clearly that negligible amounts of free phenol are formed and that the apparent diol formation by rat-liver slices varied from 18 to 124 μg./g. wet wt. tissue/hr. These rates are of the same order as those obtained by technique (*a*). Under the experimental conditions 1 g. liver tissue in the presence of naphthalene can produce about 0·1 mg. of diol/hr. If the reaction proceeded at the same rate *in vivo*, a rat weighing 200 g. with 10 g. liver would produce 24 mg. diol/day. In experiments in which the excretion of diol in urine and faeces from rats dosed with naphthalene was measured (Boyland & Wiltshire, 1952) never more than 10 mg. were found in 24 hr. The rate found in the *in vitro* experiments would therefore be more than sufficient to account for the diol excreted.

There appeared to be very little difference in the reaction according to the sex of the animal from which the tissue was derived. The mean rates of diol formation were 76 and 89 μg./g. wet wt./hr. for tissue from male and female rats respectively.

Although prior treatment with naphthalene increases the resistance of rats to naphthalene as indicated by measurements of the lethal dose of naphthalene, such treatment did not appear to change the ability of the liver to metabolize naphthalene. The rates of diol formation were 18, 54, 54, 61, 78, 94 and 114 (mean 67) μg./g. tissue/hr. for liver from a series of rats which had had ten daily doses each of 50 mg. naphthalene compared with 83 μg./g. tissue/hr. in liver tissue from untreated rats.

That the product produced from naphthalene by liver slices is 1:2-dihydroxy-1:2-dihydronaphthalene is made probable by the fact that in two experiments in which it was tested the substance yielding phenol on heating with acid was ether-soluble. Of the phenol precursor in the reaction mixtures from rats 8 and 9 (Table 2), 97 and 106% was apparently soluble in ether. The only known naphthalene metabolites which yield naphthol readily on heating with acid are 1-naphthylsulphuric acid and 1:2-dihydroxy-1:2-dihydronaphthalene; of these only the latter is extracted from neutral aqueous solution by ether. It will be noticed that the liver of rats 3–8 (Table 2) contained appreciable amounts of diol

Table 2. *Formation of a substance (probably 1:2-dihydroxy-1:2-dihydronaphthalene) which yields 1-naphthol in* N-HCl *in 15 min. at 100° by tissue slices*

(Reaction vessels contained 500 mg. wet tissue, 5 mg. naphthalene in 0·05 ml. ethylene glycol monomethyl ether. All vessels contained 10 ml. Ringer-phosphate solution pH 7·4. Temperature 37°. Gas phase $O_2$.)

| Animal | Sex | Tissue | Incubation time (min.) | Naphthol found (μg.) | | | | | | Apparent 1:2-dihydroxy-1:2-dihydronaphthalene (μg. 1-napthol formed/g. tissue/hr.) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Tissue only | | Tissue + naphthalene | | Formed | | |
| | | | | Free | 15 min. | Free | 15 min. | Free | 15 min. | |
| Rat 1 | M. | Liver | 150 | 10 | 0 | 10 | 90 | 0 | 90 | 72 |
| Rat | M. | Kidney | 150 | 0 | 0 | 0 | 36 | 0 | 36 | 29 |
| Rat | M. | Spleen | 150 | 0 | 0 | 0 | 30 | 0 | 30 | 24 |
| Rat 3 | M. | Liver | 215 | — | 84 | — | 234 | — | 150 | 84 |
| Rat 4 | F. | Liver | 180 | — | 54 | — | 78 | — | 24 | 16 |
| Rat 5 | F. | Liver | 105 | — | 90 | — | 186 | — | 96 | 110 |
| Rat 6 | F. | Liver | 120 | — | 78 | — | 126 | — | 48 | 48 |
| Rat 7 | F. | Liver | 120 | — | 84 | — | 138 | — | 54 | 54 |
| Rat 8 | F. | Liver | 120 | — | 78 | — | 180 | — | 102 | 102 |
| Rat 9 | F. | Liver | 120 | — | 10 | — | 74 | — | 64 | 64 |
| Rat 10 | F. | Liver | 120 | — | 10 | — | 116 | — | 106 | 106 |
| Rat 11 | F. | Liver | 120 | — | 18 | — | 60 | — | 42 | 42 |
| Rat 12 | M. | Liver | 120 | 0 | 0 | 6 | 80 | 6 | 80 | 80 |
| Rat 13 | M. | Liver | 120 | 6 | 0 | 12 | 72 | 6 | 72 | 72 |
| Rat 14 | M. | Liver | 120 | 0 | 0 | 6 | 90 | 6 | 90 | 90 |
| Rabbit | M. | Liver | 120 | — | 12 | — | 126 | — | 114 | 114 |
| Rabbit | M. | Kidney | 120 | — | 24 | — | 48 | — | 24 | 24 |

when incubated without naphthalene. These rats had been previously injected with naphthalene.

(3) Experiments with homogenates prepared as described under (*c*), incubated aerobically with either 100 μg. naphthalene dissolved in water in a total volume of 4 ml., or 5 mg. naphthalene dissolved in 0·05 ml. methylcellosolve added to a total volume of 10 ml., for times varying from 70 to 215 min., gave no indication of metabolism of naphthalene.

## DISCUSSION

The experiments show that isolated slices of rat or rabbit-liver can oxidize naphthalene to 1:2-dihydroxy-1:2-dihydronaphthalene although not to 1-naphthol. Although the rate of the change is slow, the oxygen used being equivalent to a $Q_{O_2}$ value of 0·1 (μl. $O_2$/mg. dry wt. liver/hr.), it is sufficient to account for the metabolic conversion of naphthalene to the diol in rats treated with naphthalene. The apparent absence of 1-naphthol as a reaction product seems remarkable in view of the fact that after naphthalene injection into rats or rabbits, more 1-naphthol is excreted than naphthalenediol. It is possible that in the incubated liver 1-naphthol is formed and immediately conjugated with glucuronic acid. The conversion of naphthalene to 1-naphthol may occur in some tissue other than liver but the few experiments carried out with spleen and kidney gave no indication that the oxidation to naphthol occurred in those tissues.

Although it had been suggested that the metabolic conversion of hydrocarbons to phenols might proceed through the stage of the corresponding diol, no conversion of a diol to a phenol has been seen in isolated liver preparations. The site of oxidation of naphthalene to naphthol and the enzymes involved in this oxidation and the oxidation to the diol still require investigation.

## SUMMARY

1. On aerobic incubation with liver slices naphthalene is apparently slowly converted to 1:2-dihydroxy-1:2-dihydronaphthalene but not to 1-naphthol.

2. The rate of the reaction *in vitro* is sufficient to account for the metabolic production of the diol in rats.

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# The Oestrogenic Activity of Bovine Colostrum

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The oestrogenic activity of human colostrum (Lacassagne & Nyka, 1934) and the undoubted importance of colostrum for the neo-natal calf led us to test bovine colostrum for oestrogenic activity.

Oestrogens found in animal tissues and body fluids during the past 23 years, in particular those in pregnant-cow bile (Pearlman, Rakoff, Cantarow & Paschkis, 1947), have always been phenolic in chemical nature, although occurring partly as water-soluble, ether-insoluble derivatives (conjugates) in the case of some urines (Cohen, Marrian & Odell, 1936; Schachter & Marrian, 1938). We therefore assumed that oestrogens in colostrum would occur either as the free phenols or as derivatives yielding the phenols on acid hydrolysis; and in either case be extractable by boiling methanol.

## EXPERIMENTAL

### *Material*

Colostrum (9·5 kg. containing 2·9% fat, 20·5% solids-not-fat) was obtained on 19 February 1951 by the complete milking of the cow Pippin 12 of the Institute herd 3 hr. after calving, and was stored at −25° until required when it was rapidly thawed at room temperature.

### *Methods of extraction*

Extraction of whole colostrum with hot methanolic NaOH was unsatisfactory owing to the difficulty of separating the small phenolic fraction from the large fatty-acid fraction. Direct ether extraction of whole colostrum at pH 7, pH 1, or when a large excess of concentrated HCl had been added was not possible because emulsions were formed.

(1) *Whole colostrum* (*free oestrogens*). *Bio-assay no.* 1. Whole colostrum (50 ml.) was refluxed for 10 min. with methanol (500 ml.), cooled to 30° and filtered. The residue was again refluxed with methanol (200 ml.) for 30 min. The combined filtrates were evaporated to dryness at reduced pressure, and saturated aqueous $NaHCO_3$ solution (100 ml.) and ether (100 ml.) added. After separation the $NaHCO_3$ phase was washed with more ether (6 × 100 ml.) and the combined ether solution, after drying over $Na_2SO_4$, evaporated to dryness. The residue was dissolved in a mixture of $CCl_4$ (90 ml.) and ether (5 ml.), according to the method of Friedgood, Garst & Haagen-Smit (1948), and this solution extracted with portions of aqueous N-KOH (100, 50, 50 ml.). The combined alkaline solution was then brought to pH 1 with conc. HCl and extracted with ether (5 × 100 ml.). The ether solution was washed with saturated aqueous $NaHCO_3$ (2 × 10 ml.), dried over $Na_2SO_4$ and evaporated to dryness. The process was then repeated on a second portion (50 ml.) of colostrum and the two final products combined.

(2) *Whole colostrum* (*conjugated oestrogens*). *Bio-assay no.* 2. The combined $NaHCO_3$ solutions from process (1) above were brought to pH 1 with conc. HCl, mixed with conc. HCl (40 ml.) and boiled for 5 min. The cooled solution was then made slightly alkaline, brought again to pH 1 and extracted with ether (5 × 100 ml.). The ether solution was washed with several 10 ml. portions of water until these were neutral, dried over $Na_2SO_4$ and evaporated to dryness.

(3) *Clarified fat* (*free oestrogens*). *Bio-assays nos.* 3 *and* 4. Clarified fat (40 g.) obtained from whole colostrum (2 l.) by the method of Aschaffenburg *et al.* (1949) was dissolved in light petroleum, b.p. 60–80° (200 ml.) and divided into two portions (50 and 150 ml.). The 50 ml. portion was extracted with N-KOH (4 × 50 ml.), the alkaline solution brought to pH 7 with conc. HCl and extracted with ether (6 × 50 ml.). This solution, dried over $Na_2SO_4$, was evaporated to dryness (bio-assay no. 3). The 150 ml. portion was extracted similarly, the reagent and solvent volumes being trebled (bio-assay no. 4).

(4) *Clarified fat* (*conjugated oestrogens*). *Bio-assays nos.* 5 *and* 6. The final aqueous solution at pH 7 from the 50 ml. light petroleum of extraction (3) was brought to pH 1, freed of dissolved ether, mixed with conc. HCl (20 ml.), boiled for 5 min., cooled, made slightly alkaline, adjusted again to pH 1 and extracted with ether (7 × 100 ml.). This solution was washed with 10 ml. portions of water until they were neutral, dried and evaporated to dryness (bio-assay no. 5).

The corresponding aqueous solution from the 150 ml. light petroleum of extraction (3) was similarly hydrolysed, quantities of reagents and solvents being trebled and the hydrolysis time increased to 15 min. (bio-assay no. 6).

(5) *Separated colostrum* (*quantitative estimation of oestrogens, free and conjugated*). *Bio-assays nos.* 7 *and* 8. Three 50 ml. samples of separated colostrum, prepared by a cream separator, were individually processed for free and conjugated oestrogens by the methods (1) and (2) (except that 20 ml. conc. HCl were used instead of 40 ml.). The three free-oestrogen concentrates were combined as were the three conjugated-oestrogen concentrates.

(6) *Separated colostrum* (*ketonic fraction of conjugated oestrogens*). *Bio-assays nos.* 9, 10 *and* 11. Separated colostrum (50 ml.), prepared by a cream separator, was processed to yield a free-oestrogen and a conjugated-oestrogen concentrate by the methods (1) and (2). The latter extract was then dissolved in ethanol (12 ml.) and acetic acid (1 ml.); Girard T reagent (0·5 g.) was then added and the solution refluxed for 30 min., cooled and added to ice-cold water containing $Na_2CO_3$ (0·88 g.) when the solution was yellow to bromothymol blue. The precipitate (*P*) was isolated by extraction with ether (5 × 50 ml.). The aqueous solution was then acidified with conc. HCl (12 ml.), left for 16 hr. at 20° and extracted with ether (5 × 50 ml.), this solution being washed with 10 ml. portions of water until they were neutral. This solution on drying and evaporation yielded ketonic fraction 1 (bio-assay no. 9).

Table 1. *Results of bio-assays for oestrogenic activity in bovine colostrum and normal milk*

(For details of bio-assays see text.)

| Bio-assay no. | Source of material | Details of colostrum or milk specimen | Extraction method | Equivalent dose (ml. colostrum or milk/mouse) | Mean uterine weights (mg.) Test group | Oil-control group | 0·02 μg. Oestradiol-17β group | 0·04 μg. Oestradiol-17β group | Activity in dose (μg. oestradiol-17β/l.) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Pippin 12 | Whole colostrum | Free oestrogens | 10 | 6·3±0·4 | 3·8±0·6 | 9·4±0·7 | 13·0±1·5 | 1·0 |
| 2 | | | Conjugated oestrogens | 10 | 21·2±2·1 | 3·8±0·6 | 9·4±0·7 | 13·0±1·5 | >8·0 |
| 3 | Pippin 12 | Clarified fat | Free oestrogens by KOH extraction | 50 | 4·7±0·3 | 3·5±0·2 | 7·8±0·5 | 15·2±1·9 | 0·12 |
| 4 | | | | 37 | 3·1±0·3 | 3·8±0·3 | 8·6±0·6 | 14·0±1·1 | N.S.* |
| | | | | 112 | 4·6±0·5 | 3·8±0·3 | 8·6±0·6 | 14·0±1·1 | N.S.* |
| 5 | Pippin 12 | Clarified fat | Conjugated oestrogens only | 50 | 5·4±0·3 | 3·5±0·2 | 7·8±0·5 | 15·2±1·9 | 0·18 |
| 6 | | | | 37 | 5·3±0·2 | 3·8±0·3 | 8·6±0·6 | 14·0±1·1 | 0·16 |
| | | | | 112 | 7·3±0·4 | 3·8±0·3 | 8·6±0·6 | 14·0±1·1 | 0·13 |
| 7 | Pippin 12 | Separated colostrum (cream separator) | Free oestrogens | 15 | 4·5±0·4 | 3·9±0·5 | 6·5±0·6 | 12·7±1·4 | N.S.* |
| 8 | | | Conjugated oestrogens | 1 | 3·9±0·3 | 3·9±0·5 | 6·5±0·6 | 12·7±1·4 | N.S.* |
| | | | | 2 | 6·0±0·4 | 3·9±0·5 | 6·5±0·6 | 12·7±1·4 | 8·0 |
| | | | | 4 | 7·2±0·5 | 3·9±0·5 | 6·5±0·6 | 12·7±1·4 | 5·5 |
| | | | | 8 | 12·8±1·0 | 3·9±0·5 | 6·5±0·6 | 12·7±1·4 | 5·0 |
| 9 | Pippin 12 | Separated colostrum (cream separator) | Conjugated oestrogens: 1st ket. frac. | 5·0 | 5·6±0·3 | 3·2±0·5 | 8·8±0·9 | 12·3±0·7 | 1·8 |
| 10 | | | 2nd ket. frac. | 5·0 | 3·5±0·4 | 3·2±0·5 | 8·8±0·9 | 12·3±0·7 | N.S.* |
| 11 | | | Non-ket. frac. | 5·0 | 7·8±0·7 | 3·2±0·5 | 8·8±0·9 | 12·3±0·7 | 3·4 |
| 12 | Normal milk | Free and conjugated oestrogens | — | 20 | 4·4±0·3 | 5·0±0·6 | 10·4±0·9 | 13·0±1·5 | N.S.* |

* N.S. = activity not significantly different from zero ($P > 0·05$).

The Girard separation described was then repeated on the ether-soluble precipitate (*P*) above yielding ketonic fraction 2 (bio-assay no. 10) and a final non-ketonic fraction (bio-assay no. 11).

(7) *Normal milk (free and conjugated oestrogens). Bio-assay no.* 12. Four 50 ml. portions of a sample of bulked milk from the Institute herd were individually processed for free and conjugated oestrogens by the methods (1) and (2), using 20 ml. conc. HCl for each hydrolysis. All eight concentrates were combined.

### *Method of bio-assay*

Each final extract was dissolved in arachis oil and its oestrogenic activity estimated by the method of Evans, Varney & Koch (1941), except that the albino mice, from the closed colony of the Agricultural Research Council Field Station, Compton, Berks, were of 8–10 g. body weight, and six mice were used per group. The uteri, after dissection, were fixed in Bouin's fluid for 72 hr., pressed dry on blotting paper and weighed.

## RESULTS

Mean uterine weights of all test and control groups of mice are given in Table 1 with standard errors of the means.

The oestrogenic activity of each colostrum specimen has, for convenience, been expressed as $\mu$g. oestradiol-17$\beta$/l., but only for no. 8, where it was possible to fulfil the conditions for strictly valid bio-assay (Emmens, 1948), could a quantitative estimate of the oestrogenic activity of separated colostrum be made, activity equivalent to 5·21 $\mu$g. oestradiol-17$\beta$/l. (3·96–6·58 $\mu$g.: 5% fiducial limits) being found. The analysis of variance of this estimation is given in Table 2. In Table 3 the results are summarized diagrammatically.

Table 2. *Factorial analysis of the dose-response line of conjugated oestrogens in separated colostrum*

(Cow: Pippin 12. Bio-assay no. 8, Table 1.)

| Source of variation | Degrees of freedom | Mean square | Variance ratio | *P* |
|---|---|---|---|---|
| Difference between samples | 1 | 0·735 | — | N.S.* |
| Linear regression | 1 | 208·86 | 38·95 | $P<0·01$ |
| Departure from parallelism | 1 | 0·482 | — | N.S.* |
| Error | 20 | 5·3682 | — | — |

* N.S. = activity not significantly different from zero ($P>0·05$).

Table 3. *Summary of oestrogenic activity of colostrum of the cow Pippin* 12

(Values in $\mu$g. oestradiol-17$\beta$/l.)

Whole colostrum (free 1·0; conjugated > 8·0)

- Clarified fat (free N.S.*; conjugated 0·15)
- Separated colostrum (free N.S.*; conjugated 5·2)
  - Ketonic fraction I (conjugated 1·8)
  - Ketonic fraction II (conjugated N.S.*)
  - Non-ketonic fraction (conjugated 3·4)

* N.S. = not significantly different from zero ($P>0·05$).

Table 4. *Oestrogenic activity of body fluids*

| Body fluid | Oestrogenic activity ($\mu$g. oestradiol-17$\beta$*/l.) | Reference |
|---|---|---|
| **Bovine** | | |
| Colostrum (free oestrogens) | 1 | Pope & Roy (this paper) |
| Colostrum (conjugated oestrogens) | 5 | Pope & Roy (this paper) |
| Pregnancy blood (free oestrogens) | 4 | Szego & Roberts (1946) |
| Pregnancy bile (free oestrogens) | 330 | Pearlman *et al.* (1947) |
| Non-pregnancy urine (free oestrogens) | 1·1 | Turner, Frank, Lomas & Nibler (1930) |
| Pregnancy urine (free oestrogens) | 50–600 | Hisaw & Meyer (1929); Nibler & Turner (1929) |
| Pregnancy urine (free and conjugated oestrogens; peak value) | 1700 | Barrie, Patterson & Underhill (1935) |
| **Human** | | |
| Colostrum (whole fluid injected) | 10 | Lacassagne & Nyka (1934) |
| Pregnancy blood (free oestrogens) | 6 | Szego & Roberts (1946) |
| Pregnancy urine (free and conjugated; peak value) | 4000 | Smith, Smith & Pincus (1938) |

* The various results have, where necessary, been converted to these units for ease of comparison.

## DISCUSSION

Table 4 shows that the oestrogenic activity of bovine colostrum is of the same order as that of human colostrum and of bovine and human pregnancy blood, but much lower than that of bile and urine in pregnancy.

The fact that most of the oestrogenic activity of bovine colostrum is found in the aqueous phase as water-soluble conjugates suggests that colostrum may serve as a vehicle for the excretion of small amounts of oestrogenic metabolites. These may, however, be significant in the health of the suckling calf. It is of interest that ketonic and non-ketonic conjugated oestrogens as found by us in bovine colostrum have also been reported in bovine blood and urine (Asdell, 1952).

The failure to detect oestrogenic activity in normal milk at a dose-level equivalent to 20 ml./mouse means that activity declines by a factor of at least 10 as the secretion changes from colostrum to normal milk. Lawson, Stroud & Williams (1945) also estimated the free oestrogens in normal cow's milk but obtained no response with doses equivalent to 756 ml. in the ovariectomized rat, a finding which agrees with our results.

## SUMMARY

1. Oestrogenic activity approximately equal in amount to that found in bovine and human pregnancy blood has been found in bovine colostrum.

2. The oestrogens occur mainly as conjugates in the aqueous phase of colostrum and are partly ketonic.

3. The conjugated oestrogens of separated colostrum from one cow were equivalent to 5·2 $\mu$g. oestradiol-17$\beta$/l.

We would like to thank Dr S. Bartlett, Head of the Feeding and Metabolism Department, National Institute for Research in Dairying, for his interest in this work. We are also greatly indebted to Mrs S. A. Simpson and Miss H. M. Grundy of the Courtauld Institute of Biochemistry, Middlesex Hospital, for carrying out the bio-assays, to the Director, the Agricultural Research Council Field Station, for the supply of mice and to Dr R. Aschaffenburg of the National Institute for Research in Dairying for preparation of the clarified fat.

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# A New Technique for the Estimation and Isolation of the Hexone Bases in Protein Hydrolysates

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The difficulties inherent in the method of Kossel & Kutscher (1900) for isolating the hexone bases from gelatin hydrolysates as phosphotungstates may be partially overcome by the direct precipitation of arginine and lysine from such mixtures as their picrates (Robson & Selim, 1952). Hydroxylysine and histidine cannot, however, be likewise precipitated from gelatin hydrolysates. To overcome this difficulty we have elaborated the present method which may be used not only for the purpose of isolating the individual hexone bases but also for estimating them in small amounts of protein.

Of the many new methods introduced in recent years for isolating amino-acids from protein hydrolysates, those based on adsorption have received much attention. Active carbon preparations capable of adsorbing amino-acids from solutions have long been known, but the main application of the use of this reagent has been the quantitative removal of the aromatic amino-acids from protein hydrolysates (Schramm & Primosigh, 1943; Tiselius, Drake & Hagdahl, 1947; and Partridge, 1949). Warburg & Negelein (1921) and others have shown that certain types of charcoal oxidize amino-acids and that such oxidation may be prevented or reduced by treatment of the charcoal with hydrogen cyanide, sodium cyanide or hydrogen sulphide. From the data collected during the present investigation it would appear that such treatment of the charcoal does not affect the results to any appreciable extent.

It is well known that picric acid is strongly adsorbed by charcoal. We have now found that arginine monopicrate and histidine dipicrate are even more strongly adsorbed than picric acid itself, 5 g. of charcoal (treated as described in the experimental section) being capable of adsorbing 1 g. of the former salt. On the other hand, the picrates of lysine, hydroxylysine and ammonia, in comparison, are not so strongly adsorbed. Certain neutral amino-acids, e.g. glycine, proline, etc., form picrates under specific conditions. Passage of such picrates through a charcoal column results in the cleavage of the salt link, the picric acid being retained by the charcoal, the amino-acid moving down and out of the column if sufficient eluate is employed. This can be shown by applying a solution of glycine picrate to a column of charcoal. On washing the column with sufficient water, all of the glycine will ultimately appear in the eluate which remains colourless, indicating that the picric acid has been retained by the charcoal.

These facts form the essential basis of the present method. It consists briefly of two stages: first, the removal of the aromatic amino-acids by the passage of the protein hydrolysate, pH 2–3, over a column of charcoal followed by treatment of the column with water until the eluate gives a negative ninhydrin reaction; secondly, the conversion of the bases of the combined filtrate and eluate, pH 7–8, into their picrates by the solution therein of several moles of picric acid (Table 1). The mixture is next passed through a column of charcoal which is then washed with water until the eluate gives a negative ninhydrin reaction. By the latter process, the picrates of the hexone bases are retained on the charcoal while the free monoamino-acids and the acidic amino-acids pass out in the eluate.

Treatment of the column with 0·5N-hydrochloric acid or sulphuric acid decomposes the adsorbed picrates, the bases appearing in the percolate and the picric acid being retained by the charcoal. In this process the less well-adsorbed hydroxylysine, lysine and ammonia appear first in the percolate and a gap, varying in volume according to the length of the column, occurs, as shown by the negative ninhydrin reaction before the more strongly adsorbed histidine and arginine are eluted. The two former bases can thus be separated from the latter and estimated by the micro-Kjeldahl and periodate techniques.

When the hydroxylysine and lysine have been removed from the column, the eluant is changed to N-hydrochloric acid or N-sulphuric acid. The resulting percolate first contains histidine, then a mixture of histidine and arginine and finally arginine. The last traces of arginine on the lowest quarter of the column are removed by boiling it with dilute acid. Histidine and arginine in the combined percolate and extracts are estimated colorimetrically.

For the purpose of isolating the individual bases in quantity the above technique is slightly varied. As is proved in the experimental section, histidine is adsorbed only as its dipicrate. To the hot protein

hydrolysate, freed from aromatic amino-acids as described above, picric acid equivalent to 1 mole for the arginine and 2 moles for the histidine present (an approximate estimation of these amino-acids having been made colorimetrically) is added. When the picric acid has dissolved the mixture is cooled and passed through a column of charcoal. The column is washed with water until all the mono-amino-acids, together with the lysine, hydroxylysine and ammonia, have passed out. The column is now treated with 0·01–0·1 N-ammonia which decomposes histidine dipicrate. The removal of the histidine from the column is followed colorimetrically. When the last stage is complete, the column is washed with N-hydrochloric acid until the eluate gives only a faint Sakaguchi reaction. At this point the lowest quarter of the column is boiled with more of the eluant to remove the last traces of arginine. The histidine and arginine, separated in this way, were identified as the diflavianate and monoflavianate respectively.

Compared with the picrates of arginine and histidine, those of lysine and hydroxylysine are only weakly adsorbed on charcoal. Several molar quantities of picric acid in excess of those theoretically required for the formation of the picrates of lysine and hydroxylysine are dissolved in the mixture now free from aromatic amino-acids, histidine and arginine. The mixture is passed through a charcoal column as before, and the column washed with water until the eluate no longer gives a positive ninhydrin reaction. The column is now treated with 0·5 N-sulphuric acid until the eluate gives a negative ninhydrin reaction. The combined eluate and washings, freed from sulphuric acid (pH 10), is concentrated and picric acid dissolved in the hot solution until the latter has a pH value of 4·5. The crystalline picrate which separated melted at 220–230° and was a mixture of the picrates of lysine and hydroxylysine. One recrystallization raised the temperature of decomposition to 261°, identical with that of lysine picrate. On cooling the concentrated mother liquor, a bright yellow crystalline picrate was obtained in excellent yield, m.p. 226–227°. Analysis confirmed that it was the picrate of hydroxylysine.

The removal of the phosphates and calcium from the charcoal before use should be thorough. Any phosphoric acid remaining will tend to decompose the picrates of the basic amino-acids. Calcium, as will be shown in a later paper, forms insoluble complex salts with monoamino-acid picrates, and these may be retained on the charcoal. Further investigation has shown that the method is generally applicable to other proteins besides gelatin and, for purposes of estimation, to much smaller quantities than those mentioned below. Further results will be reported later.

## EXPERIMENTAL

### *Materials*

Gelatin (Coignet's Gold Label Leaf) was used throughout the investigation. (N, 17·17 g./100 g. of moisture-free protein; moisture, 15·4%.)

*Picric acid.* Commercial picric acid was purified by the method of Benedict (1929).

*Charcoal.* The charcoal used in all experiments was a British Drug Houses active decolorizing charcoal. It was purified before use by the following procedure. The charcoal was boiled for 1 hr. three times with 3 N-HCl, three times with distilled water and dried in the oven at 150°. It was further boiled twice with water and finally dried at 150° for 48 hr. An aqueous extract of the final product should have a pH not less than 5 and should give negative tests for both phosphates and calcium.

### *Methods of analysis*

All N determinations were carried out by the micro-Kjeldahl procedure on samples of suitably diluted solutions containing between 0·2 and 0·5 mg. of N. Digestion lasted 20 hr.

Arginine was determined colorimetrically by the Brand & Kassell (1942) modification of the Sakaguchi reaction. All colorimetric estimations were carried out on the EEL portable colorimeter using green filter 624.

Histidine was determined colorimetrically by Macpherson's modification (1942, 1946) of the Pauly reaction.

Hydroxylysine was determined by estimating the $NH_3$ produced upon treatment of a sample of the unknown solution with alkaline periodate. The procedure employed followed closely that of Van Slyke, Hiller & MacFadyen (1941), except that the $NH_3$ produced from the reaction was first aerated into excess $H_2SO_4$ and then estimated by micro-Kjeldahl distillation.

Lysine was estimated by determining the difference between the N of the lysine-hydroxylysine fraction and the hydroxylysine N as determined above.

### *Proof that histidine is only adsorbed in the form of its dipicrate*

On applying a neutral gelatin hydrolysate, free from its basic amino-acids, and in which histidine hydrochloride (100 mg.) and picric acid (1 equiv.) had been dissolved, to a column of active charcoal (10 g.) the percolate was found to be free from histidine. When a 5% aqueous acetic acid solution, which unlike mineral acids does not decompose histidine dipicrate, was allowed to pass through the column the eluate contained histidine. Development of the column with acetic acid was continued until no more histidine was eluted. At this stage the eluate was changed to N-$H_2SO_4$. Development of the column with a sufficient volume of this solution gave a percolate containing the remainder of the histidine. This experiment showed that the histidine had been adsorbed partly as such and partly as dipicrate (cf. Ewins & Pyman, 1911).

The above experiment was repeated, but the amount of picric acid added was increased to 2 equiv. On treating the column with aqueous acetic acid the effluent did not contain any histidine. Treatment, however, with N-$H_2SO_4$ removed the histidine completely.

### *Estimation of the basic amino-acids in gelatin hydrolysates*

Gelatin (25 g.) was hydrolysed by boiling it with 160 ml. of 8N-$H_2SO_4$ for 30 hr. The insoluble humin was filtered off and the filtrate diluted to about 0·5 l. with water, and neutralized to pH 2·5 with an aqueous suspension of $Ca(OH)_2$. The $CaSO_4$ precipitate was removed and extracted twice with boiling water. The filtrate and washings were combined (2 l.) and passed through a column of active charcoal (10 g. in a glass tube of 2·5 cm. internal diam.). The colourless percolate was free from humin and from tyrosine and phenylalanine, as indicated by the negative tests with the Bernhart (1938) modified Millon-Weiss reaction and by the Kuhn & Desnuelle (1937) modified Kapeller-Adler reaction. The column was washed with 1 l. of 0·2N-$H_2SO_4$ and the acid effluent added to the main percolate. Most of the $H_2SO_4$ was removed from the combined liquors with $Ca(OH)_2$ and the remaining $SO_4^{2-}$ removed with $Ba(OH)_2$. Any Ca ions remaining in solution were removed with oxalic acid, the precipitate each time being extracted twice with boiling water. To the combined filtrate and washings (approx. 4·5 l.) picric acid (20 g.) was added, brought into solution by warming, and the mixture allowed to cool to room temperature. It was then applied to a column of charcoal (100 g., 4 × 50 cm.) at a rate of about 1 l./3 hr. The column was washed with water, until the percolate gave a negative ninhydrin reaction. Volume of eluate was about 10 l. The column was then treated with 0·5N-$H_2SO_4$ and the percolate tested with ninhydrin. The first 1·5 l. of the acid percolate gave a strong ninhydrin reaction. The following 0·5–1·0 l. gave a weak test, and finally a negative test was obtained for another 0·5 l. The percolate contained lysine and hydroxylysine. Further development with the same eluant gave an eluate which contained histidine (positive ninhydrin and modified Pauly tests). At this point the eluant was changed to N-$H_2SO_4$. The first fractions of stronger acid contained only histidine, but later fractions contained histidine and arginine. Treatment of the column with N-$H_2SO_4$ was continued until the test for arginine was very weakly positive. About 7 l. of acid solution containing histidine and arginine were collected. The column of adsorbate was then extruded and its lowest quarter separated and extracted twice with boiling N-$H_2SO_4$ to remove the last trace of arginine. The filtrate from this operation was added to the arginine-histidine eluate, made up to volume, and determinations of these two bases carried out colorimetrically.

*Estimation of lysine and hydroxylysine.* The fraction containing lysine and hydroxylysine was neutralized with $Ca(OH)_2$, the $CaSO_4$ precipitate collected, extracted with boiling water and the filtrate and extract combined. A further quantity of $Ca(OH)_2$ was added to the solution to make it strongly alkaline and the mixture concentrated *in vacuo* at a low temperature, the $NH_3$ being removed in the process. The solution was finally made up to a volume of 1 l., and samples removed for determinations (Kjeldahl) of total N.

The alkaline solution was then further concentrated *in vacuo* at a low temperature and made up to a volume of 250 ml. Samples were taken for the estimation of the periodate $NH_3$.

The results of several experiments using different quantities of picric acid are shown in Table 1.

### *Method of isolation of the bases*

Since arginine and histidine are much more strongly adsorbed on charcoal than lysine and hydroxylysine it follows that when picric acid (1 mole/mole of arginine plus 2 moles/mole of histidine) is dissolved in a protein hydrolysate, and the mixture is applied to a charcoal column, arginine and histidine will be quantitatively retained on charcoal as their picrates, while free lysine and hydroxylysine will pass unadsorbed.

Gelatin (500 g.) was hydrolysed by boiling it for 30 hr. with 3 l. of 8N-$H_2SO_4$.

*Removal of the aromatic amino-acids.* The hydrolysate was diluted with 2 vol. of distilled water and the humin removed by filtration. The pH of the mixture was raised to 2·5 by addition of a suspension of $Ca(OH)_2$ and the precipitated $CaSO_4$ collected and extracted thoroughly with boiling water. The combined filtrate and extracts (approx. 10 l.) was divided into two equal portions and each passed through a column of charcoal (60 g.; 4 cm. internal diam.). The charcoal had been previously treated with 1% HCN. The columns were washed with 0·2N-$H_2SO_4$ until a negative test for arginine was given by the effluent. The eluates from the two columns were combined and the remaining $SO_4^{2-}$ and $Ca^{2+}$ completely removed, by successive precipitation with $Ca(OH)_2$, $Ba(OH)_2$ and oxalic acid. The precipitate in each case was extracted with boiling water. The combined filtrate and extracts had a volume of about 20 l. and a pH of approximately 8.

*Removal of arginine and histidine.* Estimations showed the presence in the solution of 35·15 g. of arginine and 3·0 g. of

Table 1. *Results of the estimation of the basic amino-acids in gelatin*

(The figures emphasize that the maximum values for lysine and hydroxylysine are only achieved by using more moles of picric acid than are required for the formation of the monopicrates of these bases. The data quoted are N as % of protein N.)

| Picric acid (g.) | Charcoal (g.) | Arginine | Histidine | Lysine | Hydroxylysine |
|---|---|---|---|---|---|
| 6·6 | 40 | 15·24 | 1·05 | 1·54 | — |
| 11·4 | 65 | — | — | 3·74 | 0·46 |
| 14·0 | 75 | — | — | 4·09 | 0·75 |
| 19·0 | 90 | — | — | 4·58 | 0·84 |
| 20·0 | 100 | 15·21 | 1·01 | 4·51 | 0·84 |
| 20·0 | 100 | 15·16 | 0·97 | 4·63 | 0·89 |
| 20·0 | 100* | 15·22 | 1·04 | 4·66 | 0·89 |

* Charcoal pretreated with 1% aqueous HCN.

histidine. Picric acid (56 g., 5% excess) was dissolved in the hydrolysate, and the mixture applied to a column of HCN-pretreated active charcoal (400 g., tube 8 cm. internal diam.) at a rate of about 1·5 l./hr. The resulting colourless percolate was entirely free of arginine and histidine as indicated by negative Sakaguchi and Pauly tests. The column was washed with distilled water until the eluate gave a negative ninhydrin reaction. Volume of water used was 30 l. The picrates of arginine and histidine were ultimately worked up according to the technique described below.

To the combined percolate and washings (50 l.) picric acid (320 g.) was added and the mixture stirred until all the reagent was dissolved. The solution was divided into two equal portions and each applied to a column of HCN-pretreated charcoal (850 g., tube diam. 8 cm.), at the rate of approx. 500 ml./hr. Water was percolated through each column until the eluate gave a negative ninhydrin reaction. Each column was treated with 0·5 N-$H_2SO_4$ to elute lysine and hydroxylysine. Elution with acid was stopped when the effluent gave a negative ninhydrin reaction. About 40 l. of the acid eluate were collected from each column. The combined eluates were freed from $H_2SO_4$ by the $Ca(OH)_2$, $Ba(OH)_2$, oxalic acid technique. The various precipitates were extracted with boiling water, the combined filtrates and washings (pH 10) concentrated *in vacuo* to about 500 ml. and filtered. Solid picric acid (28·5 g.) was added in portions to the hot concentrate and dissolved by stirring until the pH of the mixture had fallen to 4·5–5·0. During the addition, crystals (lysine picrate) began to separate and when the process was complete the mixture, still hot, was semi-solid. After 2 days at 0° the mixture was filtered and the collected picrate washed first with a little ice-cold water and then with ethanol. The product, decomposition point 220–230°, was recrystallized from water (500 ml.). After 2 days at 0° the product was collected, washed with ice-cold water and ethanol and finally dried at 110°. It weighed 38·0 g. and, on heating, decomposed explosively at 261°, characteristic of lysine picrate. The yield obtained corresponded to 3·5 g. lysine/100 g. moisture-free gelatin.

The filtrates and washings from these two operations were combined, concentrated to a small volume and set aside overnight at 0°. The bright-yellow crystalline picrate which had separated was collected at the pump, washed with a little ice-cold water and finally with absolute ethanol. Dried in the oven at 110° it decomposed on heating at 226–227°. The filtrate, on further concentration, yielded a small crop of similar crystals. Hydroxylysine picrate melts at 226–227° (Martin & Synge, 1941; Sheehan & Bolhofer, 1950). A few mg. of the substance treated with alkaline periodate and dimedone gave a product which, recrystallized from ethanol, melted at 185° (melting point of the dimedone-formaldehyde condensation derivative, 189°). Admixture of the product with an authentic sample of the condensation product gave no lowering of the melting point. The total yield of pure hydroxylysine picrate obtained was 5·8 g., equivalent to 0·57 g. hydroxylysine per 100 g. moisture-free gelatin.

*Preparation of hydroxylysine monohydrochloride.* Hydroxylysine picrate (1 g.) was decomposed with hot dilute HCl and the liberated picric acid removed by extraction with successive portions of hot benzene. The acid aqueous solution was concentrated to a syrup *in vacuo*, the residue taken up in hot water, clarified with charcoal and again concentrated to a thick syrup which was taken up in hot ethanol. Pyridine was added until a slight excess was present and more hot ethanol added. On scratching the side

Table 2. *The hexone base content of gelatin hydrolysates as estimated by the present method compared with previously reported figures*

(Data quoted are N as % gelatin N.)

| Amino-acid | Value | Method | Reference |
|---|---|---|---|
| Arginine | 15·20 | Present | — |
| | 15·3 | Electrodialysis, colorimetry and alkaline digestion | Macpherson (1946) |
| | 11·6 | Electrodialysis, flavianic acid precipitation | Albanese (1940) |
| | 15·3 | Diflavianate precipitation | Vickery (1940) |
| | 14·6 | Microbiological | Hier, Graham, Freides & Klein (1945) |
| | 15·7 | Microbiological | Neuman (1949) |
| Histidine | 1·0 | Present | — |
| | 1·1 | Electrodialysis and colorimetry | Macpherson (1946) |
| | 0·76 | 3:4-Dichlorobenzenesulphonic acid precipitation | Vickery & Winternitz (1944) |
| | 0·87 | Microbiological | Stokes, Gunness, Dwyer & Caswell (1945) |
| | 1·0 | Microbiological | Neuman (1949) |
| | 1·2 | Microbiological | Hier *et al.* (1945) |
| Lysine | 4·6 | Present | — |
| | 4·9 | Electrodialysis and N difference | Macpherson (1946) |
| | 4·5 | Microbiological | Hier *et al.* (1945) |
| | 5·4 | Microbiological | Neuman (1949) |
| | 6·2 | Microbiological | Stokes *et al.* (1945) |
| Hydroxylysine | 0·87 | Present | — |
| | 1·2 | Electrodialysis-periodate | Rees (1946) |
| | 0·9 | Phosphotungstic acid precipitation, periodate estimation | Van Slyke *et al.* (1941) |

Table 3. *Amounts of lysine and hydroxylysine isolated from gelatin hydrolysates by present method compared with values previously reported in the literature*

(Values quoted are g. amino-acid/100 g. moisture-free gelatin.)

| Amino-acid | Value | Method | Reference |
|---|---|---|---|
| Lysine | 3·50 | Present | — |
| | 2·91 | Benzylidene-Cu precipitation | Turba (1948) |
| | 2·72 | Benzoyl-Cu precipitation, | Kurtz (1941) |
| Hydroxylysine | 0·57 | Present | — |
| | 0·57 | Resin exchange-alumina adsorption | Sheehan & Bolhofer (1950) |
| | 0·034 | Phosphotungstic acid precipitation, acetylation-benzoylation | Martin & Synge (1941) |
| | 0·004 | Electrodialysis, fractional crystallization of the picrate | Heathcote (1948) |

of the vessel small rod-like crystals separated. Crystallization was allowed to continue overnight at 0°. The monohydrochloride was collected, washed with absolute ethanol and recrystallized from an aqueous ethanol mixture. The dry product (0·37 g.) had m.p. 229–231°. Further recrystallization affected no change in the melting point. Sheehan & Bolhofer (1950) give 215–220°, but these workers regarded their product as partially racemized. Heathcote (1950) gives 233·5° (corr.). (Found: N, 13·97. Calc. for $C_6H_{14}O_2N_2$: HCl, 14·1%.)

### *Isolation of histidine and arginine*

The procedure is based on the fact that dilute $NH_3$ solution decomposes histidine dipicrate but not arginine picrate.

Gelatin (50 g.) was hydrolysed with $H_2SO_4$ as above. The acid was partially neutralized with an aqueous suspension of $Ca(OH)_2$ until the pH of the mixture was 2·5, and the filtered solution passed over a column of charcoal (20 g.), to remove humin and the aromatic amino-acids. The column was washed with 1·5 l. of 0·2 N-$H_2SO_4$. The remainder of the $SO_4^{2-}$ and $Ca^{2+}$ still in the hydrolysate were exactly removed by the requisite additions of $Ca(OH)_2$, $Ba(OH)_2$ and oxalic acid successively. To the combined filtrate and washings (5 l.) picric acid (5·6 g.) was added and dissolved by warming. The mixture was cooled and applied to a column of charcoal (55 g. pretreated with 1% HCN, diam. 4 cm.) at the rate of 1 l./hr. The column was developed with water until a negative ninhydrin test was obtained on the effluent solution. It was then treated with 0·1 N-$NH_3$ solution until all the histidine was washed out. The ammonia eluate (light brownish due to elution of coloured materials from the charcoal by $NH_3$) was concentrated *in vacuo*. The concentrated solution was filtered and the volume made up to about 500 ml. with water. The solution, made just acid to Congo red with $H_2SO_4$, was decolorized with 0·1 g. of charcoal. The filtrate and washings from the charcoal were concentrated *in vacuo* to about 20 ml. Flavianic acid (15 g.) was added and dissolved by heating. After standing overnight at room temperature, the yellow crystalline precipitate was collected, washed with absolute ethanol and recrystallized from a little hot aqueous flavianic acid solution. The histidine diflavianate collected, washed with absolute ethanol and dried at 110°, melted at 251° (Vickery, 1927). The column, still containing the arginine picrate, was washed with water to remove the excess of $NH_3$ and then treated with N-HCl until practically all the arginine had been eluted. Excess HCl was removed *in vacuo* and the thick syrup taken up in 500 ml. of water and neutralized with NaOH until just acid to Congo red. The hot solution was then treated with flavianic acid (7·5 g.). Arginine monoflavianate immediately separated from the solution as orange crystals with the characteristic golden yellow lustre. After 18 hr. at 0° the flavianate was collected, washed with ice-cold water and absolute ethanol and dried at 110°; yield, 8·3 g. of arginine monoflavianate decomposing at 258–259° (Kossel & Gross, 1924).

*Isolation of arginine monohydrochloride.* In another experiment (50 g. gelatin), the charcoal column still containing the arginine was treated with N-$H_2SO_4$ until most of the arginine was eluted. The arginine solution was then completely freed from $H_2SO_4$ with $Ba(OH)_2$. The filtrate and washings from $BaSO_4$ were concentrated *in vacuo* to 500 ml. The alkaline solution was neutralized to pH 6 with dilute HCl and further concentrated to a thick syrup. Arginine monohydrochloride was precipitated by slow addition of absolute ethanol with stirring. It was recrystallized by redissolving in the minimum volume of water and reprecipitating by addition of absolute ethanol. The recrystallized product weighed 2·8 g. and melted at 216–217° (Cox, 1928).

## SUMMARY

1. A new technique for the estimation and isolation of the hexone bases in protein hydrolysates is described. It possesses the advantages that it does not involve the use of any expensive reagent or the need for special equipment.

2. The present method not only makes it possible to isolate and separate the individual basic amino-acids in excellent yield and high purity, but it also provides a fraction containing all the basic amino-acids suitable for the application of any of the accepted methods of the base analysis.

3. The introduction of undesirable inorganic and organic reagents is avoided, and after the removal of the bases the hydrolysate is left in a form suitable for the isolation of monoamino- and dicarboxylic amino-acids.

4. The quantity of lysine isolated in the form of the pure picrate from gelatin hydrolysates by the present method exceeds that previously reported in the literature (Table 3).

5. The method permits the isolation of pure hydroxylysine monopicrate in a yield as high as that reported by Sheehan & Bolhofer (1950), although the procedure applied here is much more simple, and yields a product which is not racemized.

6. The new technique adopted for estimation of the bases is superior to the method of electrodialysis in that lysine is obtained separately. The estimation of this base will not therefore reflect the errors cumulating from the colorimetric estimations of arginine and histidine, as does the former method.

7. Tables comparing the results obtained in the present work with those previously reported are given.

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# Reduction of Glutathione Coupled with Oxidative Decarboxylation of Malate in Cattle Lens

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Substances containing sulphydryl (SH) groups have frequently been considered to be important for the maintenance of the clarity of the lens (see Bellows, 1944). Lens contains about 400 mg. reduced glutathione (GSH)/100 g. lens and one of the two major soluble lens proteins, β-crystallin, has SH groups in the molecule. Lens proteins show greater organ than species specificity so it seems permissible to generalize, at least for the lenses of mammals. Completely cataractous lens contains neither GSH nor any protein SH. It is this fact which has for long suggested that there is some connexion between lens clarity and SH groups, although neither glutathione nor β-crystallin has been shown to take part in any enzyme process occurring in lens. Nor has it been shown that loss of SH groups precedes rather than follows lens opacity.

Herrmann & Moses (1945) showed the presence of a system in lens which reduced oxidized glutathione (GSSG). The system was unstable and lost its activity after 3 hr. at room temperature; it could be reactivated by boiled extracts of corneal epithelium, kidney or liver but not by glucose and diphosphopyridine nucleotide (DPN). Mapson & Goddard (1951) and Conn & Vennesland (1951) have shown that GSSG is enzymically reduced by reduced triphosphopyridine nucleotide (TPNH) in plant extracts. Rall & Lehninger (1952) have demonstrated the presence of a similar reductase in animal tissues. Mapson & Goddard showed that

GSSG in pea extracts was reduced at the expense of malate or *iso*citrate in the presence of TPN and $Mn^{2+}$. It occurred to us that a similar mechanism might operate in lens and this has been found to be the case. Malate was much more effective as hydrogen donor than *iso*citrate. The reactions are coupled as follows:

$$\text{Malate}^{2-} + \text{TPN}^{+} \rightleftharpoons \text{pyruvate}^{-} + \text{TPNH} + \text{CO}_2, \quad (1)$$

$$\text{TPNH} + \text{GSSG} \rightarrow 2\ \text{GSH} + \text{TPN}^{+}. \quad (2)$$

These reactions, and the enzymes catalysing them, malic enzyme (Ochoa, Mehler & Kornberg, 1948) and GSSG reductase, have been studied in dialysed extracts of cattle lens.

## EXPERIMENTAL

### *Materials*

*Preparation of lens extract.* Cattle eyes were removed immediately after death and were usually stored in the ice chest for a few hours. Whole lenses, usually with their capsules intact, were weighed and finely chopped in a mortar with scissors. They were then ground with a little sand and twice their weight of water or 0·029 M-sodium diethylbarbiturate (veronal) acetate buffer, pH 7·25 (Michaelis, 1931). This 1 in 3 mush was dialysed overnight or for 40 hr. against running tap water, and the insoluble part was then centrifuged off. An extract of cortex was made by using only the outer soft part of the lens, plus the capsule. The weight of one lens was approx. 2·2 g., of which about half was cortex. When a comparison of cortex and nucleus was wanted, a 1 in 3 mush of the remaining hard nucleus was also made. As a general rule only the cortex was used as it was much more easily ground up.

*Adjustment of* pH *of extracts.* Extract and veronal-acetate buffer were usually mixed in the proportions in which they were to be used, the pH measured (glass electrode) and adjusted with a few drops of 0·1 N-NaOH or HCl if necessary. Phosphate buffer was not used because (*a*) it appeared to inhibit the reductase and (*b*) manganese phosphate is precipitated in the presence of $Mn^{2+}$ which was added to all malic enzyme determinations.

*Separation of α- and β-crystallins.* It has been shown by both electrophoretic and antigenic tests that the lens of many animals contains two main soluble proteins, α- and β-crystallins (Woods & Burky, 1927; Hesselvik, 1939). Together they account for about 86 % of the total lens protein. A rough separation was made by carrying out the first stage of the process used by Woods & Burky (1927). An extract was carefully brought to pH 5·2 (glass electrode) with 0·1 N-HCl. The heavy white ppt. (α-crystallin) was centrifuged down, suspended in water and dissolved by adding dilute NaOH to pH 7·25. The supernatant (β-crystallin) was also brought to pH 7·25 with dilute NaOH.

*Lens acetone powder.* Cold acetone (4 vol.) was added to a cold lens extract. The ppt. was centrifuged down and dried in a desiccator over $CaCl_2$.

*Preparation of TPN and DPN.* The method of Le Page & Mueller (1949) was used for preparing the crude nucleotides. Our product contained 21 % TPN and 27 % DPN, assuming that the $\epsilon_{340\,m\mu}$ is $5{\cdot}6 \times 10^3$. A sample of pure DPN was kindly given to us by Dr L. A. Stocken.

*Enzymic reduction of TPN and DPN.* TPNH was prepared by reducing TPN with the *iso*citric dehydrogenase in heart acetone powder (Straub, 1942), the reaction being followed in a Beckman spectrophotometer at 340 mμ. After complete reduction the solution was brought to pH 8·5, boiled for 2 min., cooled and centrifuged. It was made 0·1 N with respect to NaOH and under these conditions was stable for as least 1 week in the ice chest. Small volumes were neutralized and used as required.

DPNH was obtained by reducing DPN with alcohol dehydrogenase prepared from rabbit liver (Lutwak-Mann, 1938). A solution of the acetone powder of the liver was dialysed for 48 hr. to remove the substrates of any other enzymes.

*Preparation of* isocitric acid. Trichloromethylparaconic acid (Fittig & Miller, 1889) was converted into *iso*citric acid by the method of Krebs & Eggleston (1944).

*Preparation of GSSG.* The method of N. W. Pirie (1931) was used, except that no copper was added as catalyst.

### *Methods*

*Estimation of pyruvate and oxaloacetate.* The method of Friedemann & Haugen (1943) was used for pyruvate and that of Krebs & Eggleston (1945) for oxaloacetate.

*Estimation of GSH.* Extracts were made 2·5 % (w/v) with respect to metaphosphoric acid and titrated with 0·005 N-$I_2$. Ascorbic acid, which also reacts with $I_2$, was present in negligible amounts in dialysed extracts.

*Protein.* This was determined by the method of Warburg & Christian (1941).

### *Measurement of enzyme activity*

The activity is expressed in terms of the wet wt. of tissue. The dry wt. of adult cattle lens is about 35 % of the wet wt. (Bellows, 1944).

*Malic enzyme.* Reduction of TPN was measured at room temperature in a Beckman spectrophotometer by following the increase of optical density in a 1 cm. cell at 340 mμ.; the blank usually contained all the reagents except TPN. The system consisted of lens extract (usually 0·2 ml.), 0·029 M-veronal-acetate buffer (pH 7·25; 2·0 ml.), TPN (approx. 0·28 μmole), $MnCl_2$ (2·0 μmoles) and L-malate (3·0 μmoles), total vol. 3 ml. Readings were taken at 15 sec. intervals for 2 min. during which time the rate of reaction was practically constant. The activity was expressed as the increase in optical density ($\Delta \log I_0/I$) from 0·5 to 1·5 min.

Malate was added in the DL form, but the concentrations given in the Results section refer always to L-malate. Ochoa *et al.* (1948) have shown that D-malate is not a substrate for the malic enzyme.

*Glutathione reductase.* The decrease in optical density at 340 mμ. due to disappearance of TPNH was followed in the Beckman spectrophotometer. The blank contained all the reagents except TPNH. The system consisted of dialysed (40 hr.) lens extract (usually 0·5 ml.), 0·029 M-veronal-acetate buffer (pH 7·25; 2·0 ml.), GSSG (0·65 μmole), TPNH (approx. 0·23 μmole); total vol. 3 ml. An amount of extract was used to give a decrease of optical density between 0·005 and 0·01/min. Readings were taken every minute for 8–10 min., during which time the rate of reaction was constant. The activity was usually expressed as the decrease in optical density/min.

*Coupled reaction between malic enzyme and glutathione reductase.* The system normally comprised lens extract (2·0 ml.), 0·029 M-veronal-acetate buffer (pH 7·25; 0·5 ml.), GSSG (13·0 $\mu$moles), malate (60 $\mu$moles), $MnCl_2$ (2 $\mu$moles), TPN (approx. 0·14 $\mu$mole); total vol. 3 ml. A control omitting malate and TPN was set up at the same time and both were incubated at 37°. The GSSG was added last and 0·6 ml. samples were removed immediately and at 15 min. intervals and treated with 3 ml. of 10% (w/v) metaphosphoric acid. After mixing and centrifuging, the supernatants were kept in the ice chest until they were analysed for GSH and pyruvate.

### *Enzyme inhibitors*

With the exception of cystine, substances were incubated with the enzyme for 30 min. at room temperature before test. The effect of cystine was tested as follows: 2 ml. of 0·029 M-veronal-acetate buffer (pH 7·25) were saturated with cystine by bringing to boiling point in the presence of excess cystine and cooling to 37°. This solution was added to 2 ml. of enzyme solution at 37°. A control without cystine was prepared and both were incubated at 37° for up to 2·5 hr. After the test, both solutions were dialysed overnight against running tap water. A nitroprusside test showed a considerable diminution in the SH groups of the cystine-treated protein.

## RESULTS

### *Malic enzyme in lens*

*Presence, stability and distribution.* Dialysed lens extracts reduced TPN in the presence of malate. Fig. 1 shows that, under the conditions used, the rate of reaction was proportional to the amount of enzyme.

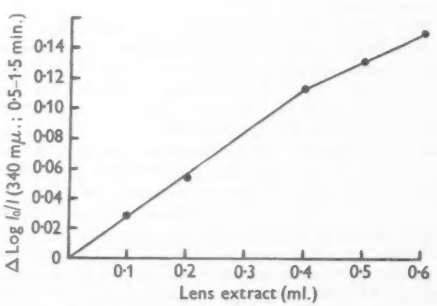


Fig. 1. Relation between malic enzyme activity and amount of lens extract. Cells contained lens extract, 0·029 M-veronal-acetate buffer (pH 7·25), 2·0 ml.; TPN, approx. 0·28 $\mu$mole; $MnCl_2$, 2·0 $\mu$moles; malate, 3·0 $\mu$moles; total vol. 3·0 ml.

Extracts of maximum activity were obtained by leaving the mush for 0·5 hr. at room temperature before centrifuging. The same amount of enzyme was extracted by both water and veronal-acetate buffer. Dialysed extracts retained full activity for about 1 week in the ice chest.

In two experiments the activity of the enzyme in the nucleus was shown to be respectively 22 and 82% more active (per unit weight of tissue) than cortex.

*Effect of* $Mn^{2+}$ *and* $Mg^{2+}$. Veiga Salles & Ochoa (1950) showed that purified malic enzyme from liver requires the presence of $Mn^{2+}$ (or $Mg^{2+}$ at higher concentrations). The activity of a dialysed lens extract was found to be slightly increased by the addition of $Mn^{2+}$. The activity of a partially purified preparation described below was more than doubled by the addition of 2 $\mu$moles manganous chloride/3 ml. Magnesium chloride at the same concentration caused a smaller increase in activity (Fig. 2).

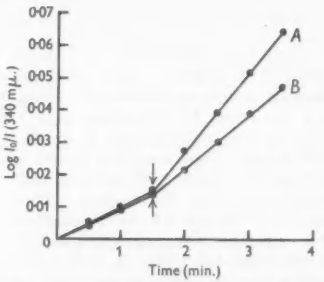


Fig. 2. Activation of malic enzyme by $Mn^{2+}$ and $Mg^{2+}$. Cells contained malic enzyme, partially purified as explained in text, 0·2 ml.; 0·029 M-veronal-acetate buffer (pH 7·25), 2·0 ml.; TPN, approx. 0·28 $\mu$mole; malate, 3·0 $\mu$moles; total vol. 3·0 ml. *A*, 2·0 $\mu$moles $MnCl_2$ added at arrow; *B*, 2·0 $\mu$moles $MgCl_2$ added at arrow.

The rate of reaction of the lens enzyme with the sample of sodium DL-malate used throughout these experiments was either slightly greater than, or equal to, the rate with a commercial sample of L-malic acid. This shows that D-malate does not inhibit the enzyme.

*Effect of* pH *and of concentration of malate and TPN.* pH-Activity curves were constructed for two different levels of malate concentration (10·0 and 1·0 mM). At the higher concentration the optimum pH was 8·2 and at the lower it was 7·4 (Fig. 3). The lower concentration was normally used because at pH 7·2–7·4 the activity was less affected by small changes in pH. Under these conditions about 0·28 $\mu$mole TPN/3 ml. were needed to ensure maximum activity for the first 2 min. There was no reduction of TPN in the absence of added malate. A sample of DPN containing no TPN was not reduced by lens in the presence of malate and $Mn^{2+}$.

*Oxaloacetate as substrate.* Like the malic enzyme of pigeon liver (Veiga Salles & Ochoa, 1948), our preparation of the malic enzyme in lens was active at pH 5·2 in decarboxylating oxaloacetate. The

preparation showed no activity with malate as substrate at this pH.

*Reversal of action.* The action of the enzyme was reversed by the addition of excess pyruvate together with carbon dioxide (Fig. 4). Addition of pyruvate alone did not bring about oxidation of the TPNH.

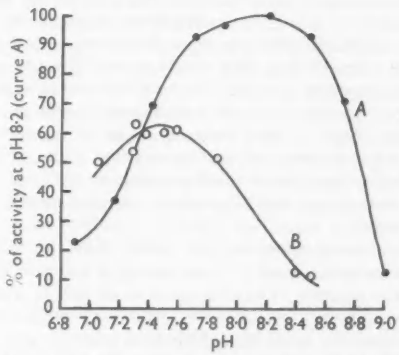


Fig. 3. pH-Activity curves of malic enzyme. *A*, 30·0 $\mu$moles malate; *B*, 3·0 $\mu$moles malate. Each cell contained: lens extract, 0·2 ml.; 0·029 M-veronal-acetate buffer, 2·0 ml.; TPN, approx. 0·28 $\mu$mole; $MnCl_2$, 2·0 $\mu$moles; malate and water to 3·0 ml. Activity was measured by change in log $I_0/I$ (340 m$\mu$.) between 0·5 and 1·5 min.

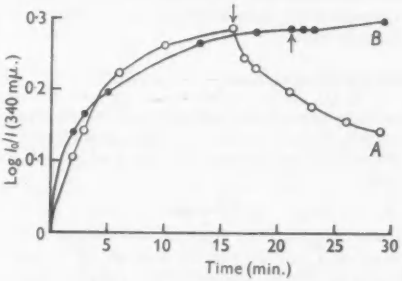


Fig. 4. Reversal of malic enzyme action by addition of pyruvate and $CO_2$. Cells contained lens extract, 0·2 ml.; 0·029 M-veronal-acetate buffer (pH 7·25), 2·0 ml.; TPN, approx. 0·17 $\mu$mole; $MnCl_2$, 2·0 $\mu$moles; malate, 3·0 $\mu$moles; total vol. 3·0 ml. *A*, 100 $\mu$moles pyruvate and 0·3 ml. 1·3% $NaHCO_3$ (saturated with $CO_2$) added at arrow; *B*, 100 $\mu$moles pyruvate added at arrow.

*Partial purification.* A preliminary attempt was made to purify the malic enzyme starting from an acetone powder (Ochoa *et al.* 1948), but an extract of the powder had only 10% of the original activity. Some purification was achieved by ammonium sulphate fractionation. An equal volume of saturated ammonium sulphate was added to an undialysed extract of lens, and after standing 15 min. the mixture was centrifuged at 8000 rev./min. for 10 min. All the activity remained in the supernatant, to which more saturated ammonium sulphate was added to bring it to 60% saturation. The resulting precipitate was centrifuged down as before and dried *in vacuo* over phosphorus pentoxide. A solution of this powder, containing 10·5 mg. protein/ml., had the same activity as the original extract which contained 100 mg./ml. The powder was unstable; it had lost about half its activity after 6 days in the ice chest. An extract from which inactive protein had been removed by 50% saturation with ammonium sulphate was no more stable than the precipitated preparation; the ammonium sulphate did not inhibit the enzyme.

Crystallins ($\alpha$ and $\beta$) are both precipitated by 50% saturation with ammonium sulphate (Krause, 1932); the filtrate contains an albumin, which accounts for about 1·5% of the total lens protein (Krause, 1933). The malic enzyme is therefore possibly associated with this albumin and can be separated from both crystallins by this means. The albumin is soluble at pH 5·2 and, as expected, most of the activity (70%) of the malic enzyme remained in the supernatant with $\beta$-crystallin when the $\alpha$-crystallin was removed by precipitation at this pH. About 8% of the activity remained in the $\alpha$-crystallin fraction but the separation of the crystallins was incomplete, as shown by the positive nitroprusside reaction given by this precipitate. Purified $\beta$-crystallin has a large number of SH groups in its molecule but $\alpha$-crystallin has none (Waters, 1950).

Table 1. *Inhibitors of malic enzyme*

(Inhibitors were incubated with the enzyme for 30 min. at room temperature before test.)

| Substance | Concn. (mM) | Inhibition (%) |
|---|---|---|
| Hydrogen peroxide | 1·0 | 60 |
| 2:4-Dinitrophenol | 1·0 | 100 |
| | 0·1 | 15 |
| Phlorrhizin | 1·0 | 100 |
| | 0·1 | 24 |
| Phenylmercuric nitrate | 1·0 | 87 |
| | 0·1 | 10 |
| *o*-Iodosobenzoate | 1·0 | 53 |
| Cupric sulphate | 1·0 | 90 |
| | 0·1 | 16 |
| Sodium fumarate | 33 | 50 |
| | 16 | 30 |
| Sodium malonate | 33 | 100 |
| | 8·3 | 55 |
| Sodium ethyl oxaloacetate (impure) | 2·0 | 90 |

*Inhibitors.* The substances which inhibit the purified enzyme are given in Table 1. The following substances did not inhibit appreciably at a concentration of 1·0 mM: *p*-aminobenzoate, cyanide,

fluoride, arsenite, arsenate, iodoacetate, azide, maleate, chloroacetophenone and thiourea. Treatment with cystine (see Methods) did not inhibit the enzyme.

We have so far assumed that the reduction of TPN is in fact due to a malic enzyme similar to that described by Ochoa *et al.* (1948), i.e. the malate is oxidized and decarboxylated according to equation 1. The evidence given is in favour of this since (i) the enzyme uses TPN and not DPN, (ii) needs $Mn^{2+}$ for full activity, (iii) is active with oxaloacetate at pH 5·2 and (iv) its action can be reversed by the addition of carbon dioxide and excess pyruvate, but not by pyruvate alone. In the next section the products of the reaction are examined by means of a coupled reaction between this enzyme and glutathione reductase.

### *Reduction of GSSG by dialysed lens extract with malate, TPN and* $Mn^{2+}$

*Reaction products.* Dialysed lens extract reduced GSSG in the presence of malate, TPN and $Mn^{2+}$ faster than in their absence. This suggested that the malic enzyme was coupling with a glutathione reductase (equations 1 and 2). The results of a typical experiment are given in Table 2. Pyruvate was formed only in the presence of malate and TPN and in amounts which agreed well with the increase in SH on the basis of equations 1 and 2 (i.e. 1 mole pyruvate/2 moles GSH).

The production of GSH from GSSG in the absence of malate and TPN is due to a reversible and probably non-enzymic reaction between GSSG and the SH groups of lens protein.

The formation of pyruvate in amounts equivalent to that of glutathione is strong evidence in favour of the occurrence of oxidative decarboxylation of malate. Furthermore, it was shown that 86% of the equivalent amount of carbon dioxide was liberated from the reaction at the same time (Table 3). The experiment was done in Warburg manometers at 37°; sulphuric acid was tipped from the side bulb after 1 hr. to stop the reaction and liberate the bound carbon dioxide. Owing to the considerable output of carbon dioxide from lens and buffer alone, 1·0 ml. only of lens extract could be used. The expected carbon dioxide production (36 $\mu$l.) was therefore small and the formation of 86% of the theoretical amount is probably as good as can be expected.

Further support for the belief that the malic enzyme is involved in these reactions was provided by the absence of oxaloacetate as an end product. This substance would be formed if dehydrogenation of malate by malic dehydrogenase were occurring.

*Effect of dialysis of extracts.* Dialysis of lens extract against running tap water for 40 hr. increased the rate of formation of pyruvate and GSH. In an experiment in which 1·4 mg. GSH were formed in 1 hr. by an undialysed extract, after dialysis 1·9 mg. GSH were formed under the same conditions. This, as shown below, was due to activation of the reductase by dialysis; there was no effect on the malic enzyme.

Table 2. *Formation of GSH and pyruvate by lens*

((i) Complete system: 2·0 ml. lens extract; 0·5 ml. of 0·029 M-veronal-acetate buffer (pH 7·25); GSSG, 13·02 $\mu$moles; sodium malate, 60 $\mu$moles; $MnCl_2$, 2·0 $\mu$moles; TPN, 0·14 $\mu$mole; total vol., 3 ml. (ii) As (i) but omitting malate and TPN. Both incubated at 37°. Samples (0·6 ml.) removed at 0, 15, 30 and 45 min. and precipitated with 3 ml. of 10% (w/v) metaphosphoric acid. GSH and pyruvate expressed as $\mu$moles/g. lens.)

| Time (min.) | GSH | | | Pyruvate | | |
|---|---|---|---|---|---|---|
| | (i) | (ii) | (i – ii) | (i) | (ii) | (i – ii) |
| 15 | 2·25 | 1·01 | 1·24 | 0·59 | 0 | 0·59 |
| 30 | 4·40 | 1·76 | 2·64 | 1·34 | 0 | 1·34 |
| 45 | 6·75 | 2·74 | 4·01 | 1·74 | 0 | 1·74 |

Table 3. *Formation of* $CO_2$, *GSH and pyruvate in lens*

(Warburg manometers; 37°; air. Complete system: 1·0 ml. dialysed lens extract; 1·45 ml. 0·029 M-veronal-acetate buffer (pH 7·25); $MnCl_2$, 2·0 $\mu$moles; GSSG, 6·5 $\mu$moles; sodium malate, 60·0 $\mu$moles; TPN, 0·056 $\mu$mole; total vol. 2·85 ml. 2 N-$H_2SO_4$ (0·15 ml.) tipped in after 1 hr. Two controls were run at the same time, one omitting malate and TPN, the other with lens and buffer alone.)

| | Complete system | Malate and TPN omitted | Lens and buffer only |
|---|---|---|---|
| Increase in pyruvate (mg.) | 0·14 | 0 | 0 |
| Increase in pyruvate due to coupled reaction (mg.) | 0·14 | — | — |
| Increase in GSH (mg.) | 1·39 | 0·35 | — |
| Increase in GSH due to coupled reaction (mg.) | 1·04 (107% of that expected from pyruvate formation) | | |
| $CO_2$ output ($\mu$l.) | 65 | 34 | 32 |
| $CO_2$ output due to coupled reaction ($\mu$l.) | 31 | (86% of that expected from pyruvate formation) | |

*Comparison of activity of cortex and nucleus.* The cortex was nearly twice as active as the nucleus in catalysing the coupled reaction. In 1 hr., 0·24 mg. pyruvic acid was formed by the cortex and 0·13 mg. by the same amount of a similar extract of nucleus. Since the malic enzyme is more active in the nucleus, this finding implies that the reductase is the limiting enzyme and is more active in the cortex. It is shown below that this is so.

*Comparison of activity under aerobic and anaerobic conditions.* The formation of GSH and pyruvate by the complete system was found to proceed at the same rate under aerobic or anaerobic conditions (Table 4). The reactions were carried out in Warburg manometers in air and in nitrogen (freed from oxygen by passing over hot copper). Rall & Lehninger (1952) measured the action of the reductase under anaerobic conditions and do not state whether their preparations were active aerobically.

Table 4. *Aerobic and anaerobic reduction of GSSG by lens*

(Warburg manometers; 37°. 1·0 ml. dialysed lens extract; 1·0 ml. 0·029 M-veronal-acetate buffer (pH 7·25); GSSG, 8·14 μmoles; malate, 60 μmoles; TPN, 0·14 μmole; $MnCl_2$, 2·0 μmoles; total vol., 2·85 ml. 2 N-$H_2SO_4$ (0·15 ml.) in side bulb, tipped in after 1 hr. to stop reaction. GSH and pyruvate expressed as μmoles/g. lens/hr.)

| | GSH formed | Pyruvate formed |
|---|---|---|
| Aerobic | 7·47 | 3·87 |
| Anaerobic | 7·67 | 3·84 |

*Lactic dehydrogenase in lens.* That the pyruvate formed is almost equivalent to the GSH in the coupled reaction suggests that lactic dehydrogenase, if present in lens, does not act rapidly through TPN. This was shown to be so; pyruvate (30 μmoles) with 0·2 ml. of dialysed lens extract oxidized TPNH (0·23 μmole) very slowly at pH 7·2. With DPNH the rate was at least 60 times as fast. The diminution in optical density in the first minute with TPNH and DPNH were 0·0027 and 0·168, respectively.

*Glutathione reductase*

The characteristics of glutathione reductase were further studied by examining the reaction between TPNH and oxidized glutathione in the absence of malate.

*Presence, stability and distribution.* TPNH was oxidized by lens extract in the presence of GSSG. Fig. 5 shows that, under the given conditions, the rate of reaction is proportional to the amount of enzyme. As with the malic enzyme, the maximum amount of reductase was extracted from crushed lens after 30 min. at room temperature and the same amount of activity was extracted by both water and veronal-acetate buffer. Active extracts retained their potency for about a week in the ice chest. (Some extracts, however, had no reductase activity.)

In two experiments the lens cortex was respectively about 2 and 4 times as active as the nucleus (wet weight basis).

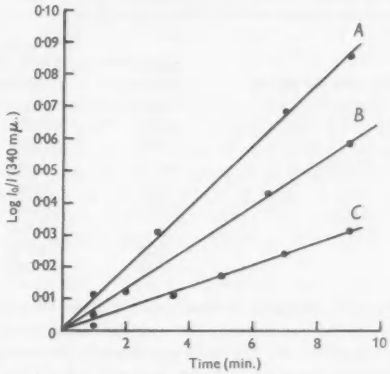


Fig. 5. Rate of oxidation of TPNH by different amounts of glutathione reductase. Each cell contained: GSSG, 0·65 μmole; TPNH, approx. 0·23 μmole; dialysed lens extract and 0·029 M-veronal-acetate buffer (pH 7·25); total vol. 3·0 ml. *A*, 0·75 ml., *B*, 0·5 ml., *C*, 0·25 ml. lens extract.

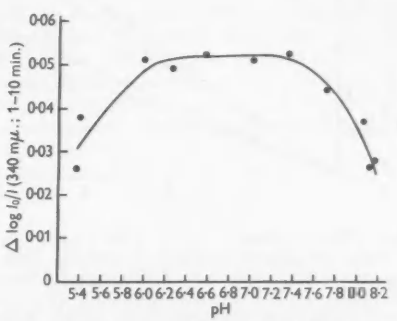


Fig. 6. pH-Activity curve of glutathione reductase. Each cell contained: dialysed lens extract, 0·5 ml.; TPNH, approx. 0·23 μmole; GSSG, 0·65 μmole; 0·029 M-veronal-acetate buffer, water to 2·0 ml.; total vol. 3·0 ml.

*Effect of* pH *and of concentrations of GSSG and TPNH.* Activity was more or less constant between pH 5·95 and 7·35 (Fig. 6). Amounts of GSSG larger than about 0·65 μmole in 3 ml. inhibited the enzyme reaction slightly (Table 5). About 0·23 μmole TPNH in 3 ml. was needed to ensure maximum activity (Table 5). DPNH at the same concentration was not oxidized. The reaction was inhibited by TPN (Table 5).

Table 5. *Effects of concentrations of GSSG, TPNH and TPN on reductase activity*

(System: 0·5 ml. dialysed lens extract; 2·0 ml. 0·029 M-veronal-acetate buffer (pH 7·25); GSSG, 0·65 μmole; TPNH, 0·23 μmole; total vol. 3 ml. Decrease of absorption of TPNH followed at 340 mμ. Blank contained all reactants except TPNH. The concentration of no more than one substance was varied in any experiment.)

| Substance added | Amount (μmole) | Decrease in optical density in 10 min. |
|---|---|---|
| GSSG | 0·32 | 0·076 |
| | 0·65 | 0·074 |
| | 0·97 | 0·066 |
| TPNH | 0·11 | 0·054 |
| | 0·23 | 0·064 |
| | 0·34 | 0·064 |
| TPN | 0 | 0·090 |
| | 0·28 | 0·067 |
| | 0·42 | 0·052 |

*Effect of dialysis.* A lens mush dialysed for 40 hr. before centrifuging was more active than the same mush kept at 4°. In two experiments the increases of activity were 40 and 80 % respectively. That this increase in activity was due to the removal by

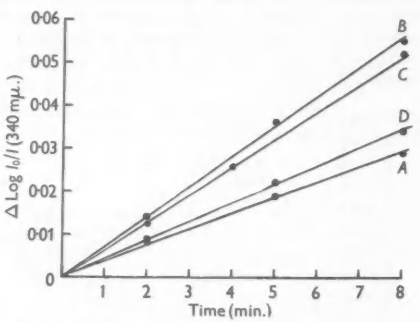


Fig. 7. Effect of dialysis and of addition of GSH and cysteine on glutathione reductase activity. Each cell contained: GSSG, 0·65 μmole; TPNH, approx. 0·23 μmole; 0·029 M-veronal-acetate buffer (pH 7·25), 2·0 ml.; total vol. 3·0 ml. The undialysed lens extract contained 255 mg. GSH/100 g. lens (i.e. 1·39 μmoles GSH/0·5 ml. 1 in 3 lens extract). *A*, 0·5 ml. undialysed lens extract; *B*, 0·5 ml. same lens extract after 48 hr. dialysis; *C*, as *B* with addition of 13·9 μmoles cysteine; *D*, as *B* with addition of 1·39 μmoles GSH.

dialysis of nearly all the GSH was shown by adding to the dialysed extract the same amount of GSH as was present in the original extract. The activity was diminished practically to its former level (Fig. 7). Cysteine did not inhibit the dialysed extract even when added at a concentration 10 times as great as that of the GSH (Fig. 7). The concentration of GSH in the cortex of cattle lens is 388–570 mg./100 g. tissue and in the nucleus 64–100 mg./100 g. tissue (Herrmann & Moses, 1945); it dialyses out of an extract rather slowly. Thus, in one extract the initial concentration of GSH was 479 mg./100 g. lens. After dialysis overnight against running tap water it was 162 mg./100 g. lens. After a further 24 hr. dialysis it was 14 mg./100 g. lens.

Ascorbic acid did not inhibit the glutathione reductase of a dialysed extract at a concentration about 20 times greater than that found in normal cattle lens (30 mg./100 g.).

*Reversal of activity.* No reduction of TPN (0·28 μmole) took place in the presence of large amounts of GSH (45·6 μmoles). This confirms the findings of Rall & Lehninger (1952) for the reductase in other animal tissues.

*Inhibitors.* The substances which inhibited the enzyme are set out in Table 6. The following substances did not inhibit appreciably at a concentration of 1·0 mM: fluoride, cyanide, arsenite, arsenate, azide, maleate, *o*-iodosobenzoate, *p*-aminobenzoate, cystine, hydrogen peroxide and thiourea. Some of the inhibitors and also some of the non-inhibitors are known to react with the SH groups in proteins. It is not therefore possible, using these impure enzyme preparations, to determine whether or not an SH group is necessary for activity.

Table 6. *Inhibitors of GSSG reductase*

(Substances were incubated with a dialysed lens extract in veronal-acetate buffer (pH 7·25) for 30 min. at 20° before test.)

| Substance | Concn. (mM) | Inhibition (%) |
|---|---|---|
| 2:4-Dinitrophenol | 1·0 | 25 |
| Phenylmercuric nitrate | 1·0 | 72 |
| | 0·1 | 40 |
| Chloroacetophenone | 1·0 | 90 |
| | 0·1 | 15 |
| Phlorrhizin | 1·0 | 100 |
| | 0·1 | 25 |
| Cupric sulphate | 0·1 | 45 |
| GSH | 2·8 | 35 |

*Purification of the reductase.* An attempt was made to purify the enzyme by ammonium sulphate fractionation as described by Conn & Vennesland (1951). The enzyme appeared to be precipitated at 40 % saturation, but after dialysis only about half the activity was recovered in the precipitate. About 55 % of the original activity was recovered in an acetone powder of lens cortex.

*Comparison between activities in α- and β-crystallin fractions.* The α- and β-crystallin fractions each contained about the same amount of reductase activity. But the sum of the activities of each fraction measured separately was greater than the activity of the two fractions combined, or that of the

original extract (Table 7). This was shown to be due to an inhibitory factor present in the original extract and precipitating in the α fraction. Thus the activity of lens extract combined with β fraction was nearly the same as the sum of their separate activities, but that of lens extract combined with α fraction was less than the sum of their separate activities (Table 7). We do not know the nature of this inhibition, but Woods, Burky & Woodhall (1933) showed that the antigenic properties also of α- and β-crystallins were not independent of each other, β-crystallin inhibiting the antigenic action of α-crystallin.

Table 7. *Glutathione reductase activity in α- and β-crystallin fractions*

(10 ml. lens extract acidified to pH 5·2; precipitate (largely α-crystallin) dissolved in water, brought to pH 7·25 and made up to 10 ml.; supernatant (largely β-crystallin) adjusted to pH 7·25 and vol. 10 ml. Activity tested as in Table 5.)

| Sample | Decrease in optical density in 10 min. |
|---|---|
| 0·5 ml. lens extract | 0·060 |
| 0·5 ml. α fraction + 0·5 ml. β fraction | 0·059 |
| 0·5 ml. α fraction | 0·044 |
| 0·5 ml. β fraction | 0·039 |
| 0·5 ml. lens extract + 1·0 ml. α fraction | 0·129 |
| 0·5 ml. lens extract + 1·0 ml. β fraction | 0·145 |

Sum of separate activities of 0·5 ml. α + 0·5 ml. β = 0·083 (141 % of activity of the two together).

Sum of separate activities of 0·5 ml. extract + 1·0 ml. α = 0·148 (115 %).

Sum of separate activities of 0·5 ml. extract + 1·0 ml. β = 0·138 (95 %).

*Relative activities of malic enzyme and reductase in lens extract*

The reduction of TPN by the malic enzyme was always faster than its oxidation by glutathione reductase. Both were measured near the physiological pH and under optimum conditions so far as is known. If the activities are expressed as change of optical density/10 min./g. tissue, the activity of the reductase in dialysed extracts ranged from 0·25 to 1·3 and that of malic enzyme from 3·2 to 9·6. The difference would have been greater had whole lenses been used, since the nucleus contains more malic enzyme and less reductase than the cortex. On the one occasion when the activities were compared in a whole lens the reductase activity was 0·15 and that of the malic enzyme 5·6.

## DISCUSSION

The metabolism of the lens is known to be sluggish compared with that of other organs. Ely (1949) found $Q_{O_2}$ 0·09 μl. oxygen/mg. dry wt./hr. for whole rabbit lens suspended in glucose saline. Merriam & Kinsey (1950) showed that, in tissue culture, a rabbit lens (weighing about 0·5 g.) used 3·4 mg. glucose in 24 hr. and formed 2·0 mg. lactic acid. We have estimated hexokinase in cattle lens by the method of Long (1952) and find that the activity is measurable but very small.

Merriam & Kinsey's results show that some of the glucose used by the lens does not appear as lactic acid and is presumably oxidized. The lens, being avascular, is dependent on the glucose of the aqueous humour for its supply of carbohydrate and on the dissolved oxygen of the aqueous humour for its respiration. Cytochrome oxidase and cytochrome *c* appear to be present in lens epithelium (Herrmann & Moses, 1945; Kinsey & Frohman, 1951) but not in the inner layers, which implies that aerobic metabolism is confined to the epithelium and cortex.

It is in the oxidation of glucose that the enzymes described in this paper may take part. The metabolites of the coupled reaction we have described are all known to be present in cattle lens, namely TPN 4·0 mg./100 g. (Euler, Hellström, Schlenk & Günther, 1939), malic acid 9·9–13·5 mg./100 g. (Krause & Stack, 1939) and pyruvate 0·61 mg./100 g. (unpublished results). The concentration of GSH is between 200 and 600 mg./100 g., depending on age and species. GSSG appears to be present only in traces, and as in other tissues the pathway of GSH oxidation is unknown. Kinsey & Merriam (1950), using radioactive glycine, have found that about 25 % of lens glutathione is renewed in 24 hr. The coupled reaction between malate, TPN and GSSG may be one way in which the high level of GSH is maintained, but the unknown factor is the mechanism of oxidation of glutathione.

## SUMMARY

1. Reduction of glutathione has been coupled through triphosphopyridine nucleotide with oxidative decarboxylation of malate in extracts of cattle lens and the distribution and properties of the two enzymes involved, malic enzyme and glutathione reductase, have been studied.

2. The malic enzyme has been shown to be of the same type as the malic enzyme of liver described by Ochoa *et al.* (1948), and the glutathione reductase is similar to that found by Rall & Lehninger (1952) in a number of different tissues.

3. The possible function of the coupled reaction in lens metabolism is discussed.

We wish to record our thanks to the National Council to Combat Blindness Inc., New York, for the Beckman spectrophotometer used in this work and also to the staff of the Oxford and District Co-operative Slaughterhouse for their help in obtaining fresh cattle eyes.

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# Studies on Sulphatases

## 1. THE CHOICE OF SUBSTRATE FOR THE ASSAY OF RAT-LIVER ARYLSULPHATASE


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The existence of arylsulphatase was first demonstrated by a colorimetric procedure when Derrien (1911), using an extract of the gastropod *Murex trunculus*, liberated indoxyl from indoxylsulphate. The colour of the indigo formed by subsequent oxidation of the indoxyl led Derrien to name the enzyme purpurase. No attempt was made to place this observation on a quantitative basis and the first measurements of arylsulphatase activity were confined to the estimation of the liberated sulphate ion by gravimetric (Neuberg & Kurono, 1923) and turbidimetric methods and potentiometric titration (Soda, 1936). Although these methods possess the inherent advantage of applicability to the assay of all sulphatases, using any of the appropriate sulphate esters, they are limited in practice by their low sensitivity. Incubation periods of up to 20 days were sometimes necessary. The benzidine (Tanaka, 1938*a*, *b*) and manometric methods (Lindahl, 1935) of sulphate estimation, as adapted to sulphatase assay, are more sensitive, but the conditions for assay under varying circumstances have not been fully established.

Attention has thus been turned towards estimation of the phenols liberated from arylsulphates, and for this purpose several colorimetric and spectrophotometric methods are available. Briefly the methods are of two types: (i) using phenols which, in the anionic form, absorb light maximally at wavelengths in the visible or ultraviolet regions of the spectrum where the corresponding ethereal sulphate has negligible absorption; (ii) using phenols which can be estimated colorimetrically after a chemical reaction which does not affect the respective arylsulphate.

A colorimetric method of type (i) was introduced by Morimoto (1937) who used a comparator to measure the yellow *p*-nitrophenol released from *p*-nitrophenylsulphate. Huggins & Smith (1947) modified the method for the Evelyn photoelectric colorimeter and later greater sensitivity was achieved for the estimation of the isomeric mononitrophenols by the use of the spectrophotometer (Robinson, Smith, Spencer & Williams, 1952). A similar colorimetric method has been developed by Robinson, Smith & Williams (1951*a*), using the red

colour of the 4-nitrocatechol anion liberated from 2-hydroxy-4-nitrophenylsulphate. In the ultraviolet range the sulphates of *p*-chlorophenol and *p*-hydroxybenzaldehyde have been used as substrates for the measurement of the arylsulphatase activity of takadiastase, the liberated phenols being estimated spectrophotometrically (Robinson *et al.* 1952).

Two methods of type (ii) have been described. Phenol released from phenylsulphate was determined after colour formation with Folin-Ciocalteu reagent (Abbott, 1947); 6-bromo-2-naphthol from its sulphate ester was coupled with tetrazotized *o*-dianisidine and the resultant dye estimated after extraction with ethyl acetate (Seligman, Chauncey & Nachlas, 1951). Robinson, Smith & Williams (1951*b*) have used the same coupling reagent with 8-hydroxyquinoline for the assay of β-glucuronidase and have suggested the similar use of 8-hydroxyquinoline as a substrate for arylsulphatase. No details have been presented.

Assay of the arylsulphatase of rat tissues has been confined to the *p*-nitrophenol (Huggins & Smith, 1947; Abbott & East, 1949), and 4-nitrocatechol methods. During repetition of this work it became apparent that the arylsulphatase of rat liver was not fully released in extracts prepared according to the direction of Huggins & Smith (1947) and Robinson *et al.* (1951*a*). Such extracts contained only a proportion of the total arylsulphatase of the liver tissue and large amounts remained in the extracted material (see Dodgson, Spencer & Thomas, 1953). Whole homogenates were therefore used, but recovery of added *p*-nitrophenol and 4-nitrocatechol from such homogenates was low. Huggins & Smith make no record of recovery experiments, and the successful recovery of 4-nitrocatechol has been attempted with tissue extracts only (R. T. Williams, private communication).

Recovery experiments of phenols used in the available arylsulphatase assay methods from whole homogenates of rat tissues have been carried out, and a search made for other phenols which can be quantitatively recovered and which are suitable for the estimation of arylsulphatase.

The colour reaction for the estimation of phenol (Abbott, 1947) is relatively insensitive and unspecific, and the procedure is laborious in comparison with other methods. The absorption maxima of *p*-chlorophenol are low and occur at wavelengths where protein absorbs strongly. (Spencer & Williams, 1950). For these reasons no attempt has been made to study the recovery of phenol or *p*-chlorophenol from rat tissues.

## EXPERIMENTAL AND RESULTS

*Animals and materials.* Rats of the Medical Research Council hooded strain were used throughout. Occasional albinos were produced in some litters. Commercially available phenols were recrystallized and melting points taken as criteria of purity. The 4-nitrocatechol (m.p. 173–175°) was prepared by the method of Benedikt (1878) with the introduction of a steam-distillation stage to remove 3-nitrocatechol and unchanged catechol. The *p*-hydroxybenzyl alcohol (m.p. 125°) was prepared according to Auwers & Daecke (1899) and 6-bromo-2-naphthol (m.p. 129–130°) according to Koelsch (1940). The isolation of *p*-hydroxyhippuric acid from human urine followed the method of Quick (1932). All melting points are uncorrected.

*Preparation of potassium* p*-acetylphenylsulphate.* The method of Burkhardt & Lapworth (1926) was modified. Diethylaniline (40 g., 43 ml.) was dissolved in 40 ml. $CS_2$ and cooled in an ice-salt mixture. Chlorosulphonic acid (13·6 g., 17·7 ml.) was added dropwise to the mixture with stirring so that the temperature did not rise above 5°. After the addition the temperature was allowed to rise to that of the room and 14·5 g. *p*-hydroxyacetophenone were added as a suspension in $CS_2$. The mixture was stirred for an hour and allowed to stand a further hour or overnight as was convenient. After removal of the lower layer of $CS_2$ and excess diethylaniline, the brown syrup of the diethylaniline salt of *p*-acetylphenylsulphuric acid was poured slowly with stirring into 30 ml. water containing 14·5 g. KOH and 4 g. $Ba(OH)_2$. Care was taken to ensure that the temperature did not rise above 35° and that the final mixture was alkaline. The sludge was filtered by suction until dry. The dry material was suspended in 250 ml. ethanol, filtered and washed well with ethanol followed by ether. Extraction of the potassium *p*-acetylphenylsulphate was achieved by boiling the washed and dried material with 35 ml. water for a few seconds and filtering after treatment with charcoal. The filtrate deposited needle crystals of the arylsulphate which were separated, dried by suction and washed well with ethanol and ether to remove contaminating $CS_2$ and diethylaniline. Recrystallization from small volumes of water was repeated (usually two or three times) until the compound was free of inorganic sulphate. A further recrystallization was made from aqueous ethanol. Analysis of the compound gave C, 37·3; H, 2·8; S, 12·1; K, 14·3. $C_8H_7O_5SK$ requires C, 37·7; H, 2·8; S, 12·6; K, 15·4%. The compound was free from $Cl^-$ and $SO_4^{2-}$ but contained small traces of the parent phenol ($\epsilon_{max.}$ 160). This state of purity is sufficient for most enzyme studies. Four further recrystallizations from aqueous ethanol gave a product apparently free from *p*-hydroxyacetophenone since the molecular extinction coefficient at 323 mμ. in 0·1 N-NaOH did not decrease on further recrystallization.

### *Spectrophotometric estimation of phenols added to tissue homogenates*

A standard procedure, based on that of Spencer & Williams (1951) for *p*-chlorophenol, was used for the estimation of *p*-nitrophenol, *p*-hydroxybenzaldehyde, 4-nitrocatechol and *p*-hydroxyacetophenone. Tissue homogenate, buffer and phenol were incubated together, 'deproteinized' with ethanol (final concentration 80%) and alkali added to convert the phenol into the anionic form. The phenol was then measured spectrophotometrically at the wavelength of its maximum absorption. Controls were prepared in a similar manner but omitting the phenol.

Table 1. *Absorption spectra of several phenols*

| Phenol | Solvent: 0·1 N-NaOH $\lambda_{max.}$ (mμ.) | 0·1 N-NaOH $\epsilon_{max.}$ (mμ.) | 66·7% Ethanolic NaOH (0·167 N) $\lambda_{max.}$ (mμ.) | 66·7% Ethanolic NaOH (0·167 N) $\epsilon_{max.}$ (mμ.) | 0·1 N-HCl $\lambda_{max.}$ (mμ.) | 0·1 N-HCl $\epsilon_{max.}$ (mμ.) |
|---|---|---|---|---|---|---|
| *p*-Nitrophenol | 227·5 | 5 850 | 228 | 6 200 | 226 | 7 100 |
| | 400·0 | 18 200 | 402·5 | 21 000 | 315·5 | 9 900 |
| 4-Nitrocatechol* | 510 | 11 200 | 510 | 11 200 | — | — |
| *p*-Hydroxybenzaldehyde | 237·5 | 7 680 | 241 | 7 350 | 218 | 11 600 |
| | 330 | 27 500 | 335 | 30 200 | 285 | 16 000 |
| *p*-Hydroxyacetophenone | 235 | 5 700 | 238 | 6 000 | 218·5 | 8 600 |
| | 323 | 20 500 | 327·5 | 21 700 | 274 | 12 450 |

* Measured in the visible range only.

Table 2. *Conditions for the measurement of recovered phenols*

(For definition of *F* see text.)

| Phenol | Light source | Slit width (mm.) | Wavelength (mμ.) | *F* |
|---|---|---|---|---|
| *p*-Nitrophenol | Filament | 0·3 | 402·5 | 46·33 |
| 4-Nitrocatechol | Filament | 0·16 | 510 | 96·81 |
| *p*-Hydroxybenzaldehyde | Hydrogen | 0·5 | 335 | 28·28 |
| *p*-Hydroxyacetophenone | Hydrogen | 0·5 | 327·5 | 43·86 |

The final solution contained approximately 66·6% (v/v) ethanol and was 0·167 N with respect to sodium hydroxide. The absorption spectra of some phenols under these conditions differ slightly from those observed in aqueous sodium hydroxide. The data are presented in Table 1. The spectra of the unchanged form of the phenols are included since they approximate closely to those of the corresponding *O*-conjugate in both hydrochloric acid and sodium hydroxide (see Discussion). All absorption spectra were determined with a Hilger 'Uvispek' spectrophotometer and the quartz prism. Quartz cells, 1 cm. optical length, were used throughout.

*Estimation procedure.* The fresh rat tissue was homogenized in 0·5 M-acetate buffer at the required pH with a glass homogenizer (Potter & Elvehjem, 1936) or a macerator similar in design to that of Campbell & Davidson (1949). The method of tissue preparation did not appear to influence the recoveries of the phenols. The results reported below are those obtained with homogenates prepared by grinding the freshly removed wet tissue with the buffer for 2 min. in a glass homogenizer under ice-cold conditions.

The homogenate (0·6 ml.) was added to each of four 15 ml. centrifuge tubes which were then placed in a water bath at 37·5°. After 4 min. the phenol, dissolved in 0·6 ml. 0·5 M-acetate buffer at the same pH as the homogenate, was added to two of the tubes (test) and 0·6 ml. of the buffer without the phenol added to the remaining two tubes (control). The solutions were mixed and the tubes stoppered and incubated for the requisite period. After the addition of 4·8 ml. ethanol at the end of the incubation period the tubes were sealed with rubber caps and centrifuged to remove protein. A 5 ml. portion of the clear supernatant of each tube was added to 1 ml. N-NaOH and the absorption at the wavelength of maximum absorption (see Table 2) measured against an ethanol blank. In the case of 4-nitrocatechol the period between the addition of ethanol and the measurement of the absorption was less than 10 min., since the colour faded after this time due to atmospheric oxidation.

The amount of phenol present in the incubation mixture is given by

$$\mu\text{g. phenol} = \frac{(E_t - E_c) \times \text{mol.wt.} \times 5{\cdot}88 \times 5{\cdot}94 \times 10^6}{\epsilon_{max.} \times 5 \times 10^3},$$

or

$$\mu\text{g. phenol} = (E_t - E_c) \times F,$$

$E_t$ and $E_c$ are the observed extinctions of test and control solutions and $\epsilon_{max.}$ the molar extinction coefficient in 66·6% (v/v) ethanolic NaOH (0·166 N) (Table 1). Values of *F* are given in Table 2. The numbers 5·88 and 5·94 occurring in the equation differ from 6·00 because of the shrinkages of volume which occur on mixture of 4·8 ml. ethanol and 1·2 ml. incubation mixture, and on mixture of 5 ml. of this solution with 1 ml. N-NaOH. At the wavelengths of maximum absorption of the respective phenols the Lambert-Beer Law appeared to be obeyed in the range of log $I_0/I$ 0·050–1·500 for the four phenols under the conditions of the experiment.

*Estimation of 6-bromo-2-naphthol.* The improved method using $CHCl_3$ for extraction of the dye was employed (Cohen, Tsou, Rutenburg & Seligman, 1952). Stabilized tetrazotized *o*-dianisidine is available in this country under the trade name of Brentamine Fast Blue B Salt (Imperial Chemical Industries Ltd.). The incubation mixture was composed of 0·6 ml. homogenate in acetate buffer and 5 ml. water containing the 6-bromo-2-naphthol. Controls without 6-bromo-2-naphthol were run simultaneously.

*Recovery experiments*

Recoveries were tested from pure solution, and from solutions containing up to 2 g. wet wt. tissue/100 ml. homogenate with and without incubation. Recovery from pure solution was in all cases $100 \pm 1\%$. From unincubated solution of homogenate, recoveries were similarly complete, except in the case of 6-bromo-2-naphthol: here only 70–80% was recovered, owing to absorption of the coupled dye on the trichloroacetic precipitate (cf. Cohen *et al.* 1952). Incubation did not further reduce the recovery of 6-bromo-2-naphthol and recoveries of the other phenols after incubation were $100 \pm 2\%$, except where liver tissue was used: here lower recoveries were obtained depending on the time of incubation (Table 3).

Recoveries of *p*-nitrophenol, 4-nitrocatechol and 6-bromo-2-naphthol, were checked at the pH used in the original assays of rat tissue arylsulphatase (Abbott & East, 1949; Robinson *et al.* 1951*a*; Seligman *et al.* 1951). In the cases of *p*-hydroxybenzaldehyde and *p*-hydroxyacetophenone the optimum pH of rat arylsulphatase acting on the sulphate esters of these phenols was not known and recoveries were made in the pH range 6·0–7·2. Later work (Dodgson *et al.* 1953) has shown that the optimum pH of rat liver arylsulphatase acting on potassium *p*-acetylphenylsulphate in acetate buffer is 7·2.

*Explanation of low recoveries of* p*-nitrophenol and* p*-hydroxybenzaldehyde*

*Spectroscopic examination of incubation solutions after deproteinization.* An examination of the spectrum of a *p*-nitrophenol 'test solution', prepared in the manner detailed in the estimation procedure, gave an indication of the fate of the *p*-nitrophenol lost after incubation with rat-liver homogenates. The spectrum in Fig. 1 is that of 37·9 $\mu$g. *p*-nitrophenol after incubation with a 3·5% rat-liver homogenate for 2 hr. at pH 6·0 measured against a 'control solution' in the blank cell. There was a 16·9% loss of *p*-nitrophenol. The spectrum shows a small peak at 290–295 m$\mu$. which is absent from the spectrum of the *p*-nitrophenol anion in pure solution and after recovery from rat-liver homogenates without incubation. A possible metabolite, *p*-aminophenol, has an anionic absorption maximum in ethanol at 287 m$\mu$. (Morton & McGookin, 1934).

A similar spectroscopic examination was made of 22·8 $\mu$g. *p*-hydroxybenzaldehyde after a 2 hr. incubation with a 2% liver homogenate at pH 6·6

Table 3. *Recovery of phenols from rat-tissue homogenates after incubation*

| Tissue | Time of incubation (hr.) | Tissue in incubated mixture (%) | pH of incubated mixture | Phenol added ($\mu$g.) | Recovery (%) |
|---|---|---|---|---|---|
| *p*-Nitrophenol | | | | | |
| Liver | 1 | 2·0 | 6·6 | 19·6 | 88·0 |
| Liver | 2 | 2·0 | 6·6 | 19·6 | 80·7 |
| Intestine | 2 | 2·0 | 6·6 | 37·9 | 100·8 |
| Kidney | 2 | 1·5 | 6·6 | 37·9 | 99·4 |
| Spleen | 2 | 1·5 | 6·6 | 37·9 | 101·3 |
| Muscle | 2 | 2·0 | 6·6 | 37·9 | 100·5 |
| 4-Nitrocatechol | | | | | |
| Liver | 1 | 2·1 | 6·6 | 30·0 | 90·2 |
| Liver | 2 | 2·1 | 6·6 | 30·0 | 87·7 |
| *p*-Hydroxybenzaldehyde | | | | | |
| Liver | 2 | 2·2 | 7·2 | 18·3 | 41·0 |
| Liver | 2 | 2·0 | 6·6 | 22·0 | 58·9 |
| Liver | 1·5 | 1·5 | 6·0 | 21·7 | 90·8 |
| Kidney | 1·5 | 1·5 | 6·0 | 21·7 | 98·2 |
| Spleen | 1·5 | 1·5 | 6·0 | 21·7 | 99·2 |
| Muscle | 1·5 | 2·0 | 6·0 | 21·7 | 102·4 |
| *p*-Hydroxyacetophenone | | | | | |
| Liver | 1 | 2·2 | 7·2 | 19·6 | 100·0 |
| Liver | 3 | 2·1 | 7·2 | 28·9 | 99·6 |
| Liver | 2 | 2·0 | 6·6 | 37·1 | 98·3 |
| Liver | 2 | 3·5 | 6·0 | 37·1 | 100·2 |
| Kidney | 3 | 2·4 | 7·2 | 28·9 | 98·6 |
| Spleen | 3 | 2·0 | 7·2 | 28·9 | 100·7 |
| Brain | 3 | 3·0 | 7·2 | 28·9 | 98·3 |
| Intestine | 3 | 2·5 | 6·0 | 37·1 | 100·7 |

resulting in a 46·1% loss. The spectrum showed a small flat peak in the region 275–280 mμ. which is absent from the spectrum of pure *p*-hydroxybenzaldehyde (Fig. 1). Possible metabolites of *p*-hydroxybenzaldehyde which absorb in this region are *p*-hydroxybenzoic acid, its conjugates and *p*-hydroxybenzyl alcohol (see Table 5).

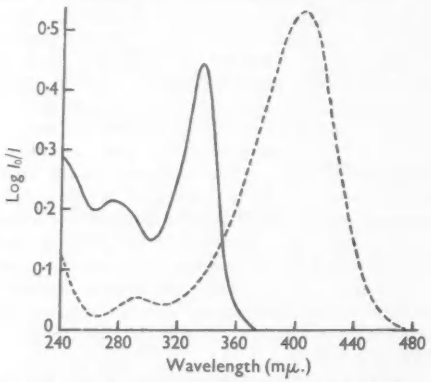



Fig. 1. Absorption spectra of solutions of *p*-hydroxybenzaldehyde (——) and *p*-nitrophenol (– – –) after incubation with rat-liver homogenates. Incubation mixtures were deproteinized with ethanol and then made alkaline with NaOH.

*Paper chromatography.* Extracts of solutions of *p*-nitrophenol and *p*-hydroxybenzaldehyde after incubation with liver homogenates were examined chromatographically for the presence of metabolites.

A 10% homogenate of rat liver in 0·5M-acetate buffer, pH 6·6 (10 ml.), was incubated with four 5 ml. portions of buffer, two of which contained *p*-nitrophenol (324 μg.) and *p*-hydroxybenzaldehyde (107 μg.) respectively. After 3 hr. the *p*-nitrophenol mixture and one of the control tubes were adjusted to pH 7·5 with M-acetate whilst the *p*-hydroxybenzaldehyde mixture and the other control were made acid to Congo red with a few drops of N-HCl. All four solutions were extracted with 25 ml. ether, centrifuged to disperse the emulsion and the ether layer separated, dried and concentrated. Quantitative experiments carried out simultaneously indicated that there was a 46% loss of *p*-nitrophenol and an 83% loss of *p*-hydroxybenzaldehyde.

The *p*-nitrophenol-homogenate extract and reference compounds were chromatographed using wet *iso*propyl ether and Barcham-Green no. 401 paper (Robinson, Smith & Williams, 1951*c*). After equilibration overnight and a 3 hr. run the dried chromatogram was sprayed with a mixture of 5 ml. N-HCl and 2·5 ml. 5% (w/v) $NaNO_2$ followed by 5% (w/v) thymol in 20% (w/v) $Na_2CO_3$. The presence of both *p*-nitrophenol and *p*-aminophenol in the extract was established; the former appearing as a yellow spot at the solvent front and the latter as a blue spot ($R_F$ 0·60). The control homogenate extract gave no spots.

The *p*-hydroxybenzaldehyde-homogenate extract was chromatographed on Whatman no. 1 paper along with reference spots of *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, *p*-hydroxybenzyl alcohol and *p*-hydroxyhippuric acid. After equilibration overnight the paper was run for 3 hr. with the non-aqueous phase of benzene:acetic acid:water (40:40:20). The spots were developed with alkaline $KMnO_4$ or the diazotized *p*-nitroaniline reagent of Bray, Thorpe & White (1950).

The results of the experiment are given in Table 4 and indicate that *p*-hydroxybenzaldehyde is oxidized in part to *p*-hydroxybenzoic acid and reduced to *p*-hydroxybenzyl alcohol. No *p*-hydroxyhippuric acid was detected. Confirmatory evidence of the composition of the spots from the homogenate extract was obtained spectrophotometrically. The spots were eluted from the paper with 0·1N-sodium hydroxide and examined against a 0·1N-sodium hydroxide blank. A portion of the eluate was made 0·1N with respect to hydrochloric acid and spectrophotometrically examined against a suitable blank. The results shown in Table 5 confirm the identity of the metabolites of *p*-hydroxybenzaldehyde as the corresponding acid and alcohol. No *p*-hydroxybenzyl alcohol could be detected in chromatograms of extracts of *p*-hydroxybenzoic acid after incubation with liver homogenates.

*Quantitative estimation of* p-*aminophenol.* No metabolite other than *p*-aminophenol was detected after incubation of *p*-nitrophenol with rat-liver homogenate. It was of interest, therefore, to

Table 4. *$R_F$ values of* p-*hydroxybenzaldehyde and related phenols*

(For conditions see text.)

| Phenol | $R_F$ | Colour with alkaline $KMnO_4$ | Colour with diazotized *p*-nitroaniline |
|---|---|---|---|
| *p*-Hydroxybenzaldehyde | 0·370 | Yellow | None |
| *p*-Hydroxybenzoic acid | 0·330 | Yellow | Red |
| *p*-Hydroxybenzyl alcohol | 0·150 | Yellow | Red |
| *p*-Hydroxyhippuric acid | 0·000 | Yellow | Red (faint) |
| Extract of *p*-hydroxybenzaldehyde after incubation with rat-liver homogenate | 0·370 | Yellow | None |
| | 0·330 | Yellow | Red |
| | 0·150 | Yellow | Red |
| Homogenate control | None | None | None |

Table 5. *Comparison of the spectra of spots eluted from paper chromatograms with those of* p-*hydroxybenzyl alcohol and* p-*hydroxybenzoic acid*

| | Solvent | | | |
|---|---|---|---|---|
| | 0·1 N-NaOH | | 0·1 N-HCl | |
| Phenol | $\lambda_{max.}$ (mμ.) | $\epsilon_{max.}$ (mμ.) | $\lambda_{max.}$ (mμ.) | $\epsilon_{max.}$ (mμ.) |
| Spot $R_F$ 0·150 | 243 | — | 222 | — |
| | 290 | — | 272 | — |
| *p-Hydroxybenzyl alcohol | 243 | 11 820 | 223 | 7 950 |
| | 290 | 2 590 | 273 | 1 360 |
| Spot $R_F$ 0·330 | 280 | — | 255 | — |
| p-Hydroxybenzoic acid | 281 | 13 400 | 255 | 14 000 |

* Moir (1923) gives $\lambda_{max.}$ 293 mμ.

establish the proportion of the loss of *p*-nitrophenol that could be accounted for by the reduction.

Acetate buffer (2·5 ml., 0·5 M, pH 6·6) containing 152 μg. *p*-nitrophenol was incubated for 2 hr. with 5 ml. of a 6% rat-liver homogenate in the same buffer. The incubation mixture was then centrifuged. A portion of the supernatant (1·2 ml.) was deproteinized with 4·8 ml. ethanol and the *p*-nitrophenol content estimated spectroscopically. Another portion (5 ml.) was assayed for *p*-aminophenol by the indophenol method detailed by Robinson *et al.* (1951*c*) using 8 ml. $K_3Fe(CN)_6$.

Suitable control determinations were made. The results showed that there was a 44 μg. loss of *p*-nitrophenol of which 42·1 μg. could be accounted for as *p*-aminophenol. Further experiments confirmed that *p*-nitrophenol was quantitatively reduced to *p*-aminophenol (94–100%, five determinations).

### *Potassium* p-*acetylphenylsulphate*

Recoveries of *p*-hydroxyacetophenone from various rat-tissue homogenates were complete, and the phenol method seemed applicable to arylsulphatase assay. It remained to be established that the absorption of potassium *p*-acetylphenylsulphate was sufficiently low at the wavelength of maximum absorption of *p*-hydroxyacetophenone (323 mμ. in sodium hydroxide, 327 mμ. in ethanolic sodium hydroxide) to allow small quantities of the phenol to be estimated in the presence of relatively large amounts of the sulphate. In 0·1 N-sodium hydroxide, potassium *p*-acetylphenylsulphate has $\lambda_{max.}$ 252·5 mμ., $\epsilon_{max.} = 11450$, whilst at 323 mμ. $\epsilon_{323} = 70$ (Fig. 2). In a solution in 0·167 N-sodium hydroxide containing 66·7% (v/v) ethanol, the sulphate had $\epsilon_{327.5} = 75$. These values indicate that the sulphate of *p*-hydroxyacetophenone may be of use as a substrate for arylsulphatase assay.

## DISCUSSION

*The unsuitability of various substrates for rat-liver arylsulphatase.* Various colorimetric and spectrophotometric methods of arylsulphatase assay have been reported. They are based on the estimation of phenols liberated from the corresponding ethereal sulphates after incubation with the source of the enzyme, and it is essential in such methods that the recoveries of the phenols from the enzyme extracts should be quantitative. In the study of rat arylsulphatase whole homogenates of the tissues must

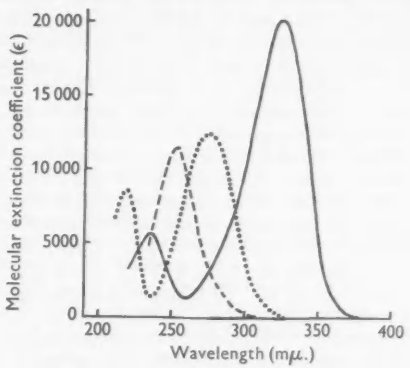



Fig. 2. Absorption spectra of *p*-hydroxyacetophenone (——) and potassium *p*-acetylphenylsulphate (– – –) in 0·1 N-NaOH and *p*-hydroxyacetophenone in 0·1 N-HCl (·····).

be used in order to obtain the full activity of the enzyme. The present work shows that recovery of *p*-nitrophenol, *p*-hydroxybenzaldehyde, 4-nitrocatechol and 6-bromo-2-naphthol from whole homogenates of rat liver is incomplete and the methods of assay using these phenols are therefore unsuitable in these circumstances.

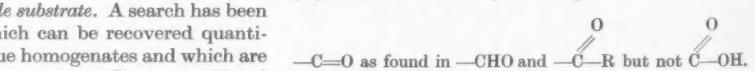
The evidence presented here suggests that the low recovery of *p*-nitrophenol can be accounted for by the formation of *p*-aminophenol. It is possible that the similar loss of 4-nitrocatechol may also be attributed to the reduction of the nitro group since the reduction of nitro groups by tissue preparations *in vitro* appears to be a reaction common to most

aromatic nitro compounds. Parker (1952) has followed the reduction of 2:4-dinitrophenol and the isomeric aminonitrophenols by rat-liver homogenates and has investigated the reaction more fully.

The action of rat liver on *p*-hydroxybenzaldehyde *in vitro* results in the formation of *p*-hydroxybenzoic acid and *p*-hydroxybenzyl alcohol. The production of these metabolites is easily explained by the action of aldehyde mutase, but Racker (1949) has produced evidence which suggests that the mutase activity is due to two separable enzymes, aldehyde oxidase and aldehyde reductase (alcohol dehydrogenase), both of which are present in liver. Aldehyde oxidase, although not able to oxidize benzaldehyde, shows some activity towards *o*-hydroxybenzaldehyde (Racker, 1949), and it is possible that it attacks the para isomer as well. The formation of *p*-hydroxybenzoic acid could also be brought about by xanthine oxidase, but no attempt has been made here to determine the enzyme responsible for the oxidation. Alcohol dehydrogenase has been reported to attack aromatic alcohols at an insignificant rate and the reverse reaction, the reduction of aldehyde to alcohol, does not appear to have been studied in the aromatic series. Furthermore, after feeding aromatic aldehydes to animals only oxidation products of the ingested compounds have been isolated from the urine (see Williams, 1947). The present study shows, however, that *p*-hydroxybenzaldehyde, but not *p*-hydroxybenzoic acid, is considerably reduced by rat-liver homogenates. If this activity is attributed to aldehyde reductase the present limits of specificity of this enzyme must be widened to include the aromatic aldehydes. Although large percentages of the *p*-hydroxybenzaldehyde incubated with liver homogenates were oxidized to *p*-hydroxybenzoic acid the formation of *p*-hydroxyhippuric acid was not observed (cf. Cohen & McGilvery, 1948).

*The choice of suitable substrate.* A search has been made for phenols which can be recovered quantitatively from rat-tissue homogenates and which are suitable for arylsulphatase assay. It was considered that the estimation of a phenol as its anion by physical means was to be preferred to methods involving chemical reaction. The selection of a suitable phenol was assisted by the observation of certain empirical relationships between the structure of phenols, their *O*-conjugates and their spectra. The absorption maxima of phenols move to longer wavelengths on changing the ionic state by addition of alkali. Thus the phenol itself has $\lambda_{max.}$ 210·5 and 270 m$\mu$., whilst the anion absorbs maximally at 235 and 287 m$\mu$. (Doub & Vandenbelt, 1947). The spectra of para-substituted phenols with substituent groups which possess neither a considerable characteristic absorption nor form a unique chromophore with the aromatic ring are similar to those of phenol. An *O*-conjugate of the phenol may be regarded as a suitable substrate for the assay of the corresponding hydrolase if its absorption is sufficiently low at the wavelength of maximum absorption of the phenol anion to permit the estimation of small quantities of the phenol in the presence of large amounts of its conjugate. An indication of the suitability of the *O*-conjugate may be obtained by examination of the spectrum of the corresponding phenol. If the compound which forms the *O*-conjugate of the phenol does not possess a characteristic absorption the spectrum of the conjugate in acid or alkali will approximate closely to that of the corresponding phenolic cation. Such a correlation exists for *p*-chlorophenol and its glucuronide (Spencer & Williams, 1951) and sulphate (Robinson *et al.* 1952). The glucosides, glucuronides, aliphatic esters, phosphates and sulphates of phenols exhibit this behaviour.

Monosubstituted phenols whose substituent groups make no major contribution to the spectra seldom have anionic maxima above 300 m$\mu$. and their measurement is thus susceptible to interference by protein which absorbs strongly below this wavelength (cf. Goodwin & Morton, 1946). Convenient deproteinization procedures, especially with crude homogenates, are often incomplete and fail to reduce protein absorption to a satisfactory level. The use of phenols having maxima above 310 m$\mu$. avoids the necessity for deproteinization, and protein precipitation need only be used for clarification of homogenates when necessary. Monosubstituted phenols with anionic maxima above 310 m$\mu$. are those in which the substituent group considerably influences the normal spectrum of phenol. Two such types of groups are (i) nitro group, (ii) a group containing a double bond which can exist with those of the aromatic ring as a conjugated double bond system, e.g.




Thus *p*-nitrophenol has $\lambda_{max.}$ 405 m$\mu$. in alkali and the anionic form of *p*-hydroxybenzaldehyde has $\lambda_{max.}$ 330. The similarity between the spectra of phenols of the two types mentioned above and those of their *O*-conjugates decreases as the substituent groups of the phenols contribute more to the spectra.

Unfortunately, the nitro and aldehydic groups are easily attacked by animal tissues *in vitro* and are therefore unsuitable chromogens. A rapid survey of the fate of phenols, which the general considerations above have suggested as suitable for the assay of hydrolases, showed that phenolic ketones appear to be resistant to attack other than

conjugation by the animal body. This class of compounds is excreted unchanged or conjugated as the glucuronide or ethereal sulphate whilst the ketone group remains intact (see Williams, 1947). The isomeric monohydroxyacetophenones were therefore considered. The para isomer was selected for study since its anionic maximum at 323 m$\mu$. has a greater molecular extinction coefficient than those of the ortho and meta compounds at 349 and 359 m$\mu$. (Doub & Vandenbelt, 1949). Since the results reported in this paper show that rat-tissue homogenates do not attack *p*-hydroxyacetophenone, and since potassium *p*-acetylphenylsulphate has very low absorption at 323 m$\mu$., the latter is a suitable substrate for arylsulphatase assay. Other *O*-conjugates of *p*-hydroxyacetophenone may be suitable substrates for the assay of the corresponding hydrolases.


## SUMMARY

1. Recovery of *p*-nitrophenol, 4-nitrocatechol and *p*-hydroxybenzaldehyde was complete from all rat tissues tested except liver. Absorption by protein of coupled 6-bromo-2-naphthol accounted for low recoveries of this phenol.

2. Rat-liver homogenates reduced *p*-nitrophenol to *p*-aminophenol whilst *p*-hydroxybenzaldehyde was metabolized to *p*-hydroxybenzyl alcohol and *p*-hydroxybenzoic acid.

3. Rat-tissue homogenates including liver did not appear to metabolize *p*-hydroxyacetophenone.

4. Potassium *p*-acetylphenylsulphate is suggested as a suitable substrate for a spectrophotometric method of arylsulphatase assay.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# Studies on Sulphatases

## 2. THE ASSAY OF THE ARYLSULPHATASE ACTIVITY OF RAT TISSUES

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The discovery of arylsulphatase activity in certain gastropods (Derrien, 1911) and extracts of *Aspergillus oryzae* (Neuberg & Kurono, 1923) was quickly extended to mammalian sources when Neuberg & Simon (1925) showed that liver, kidney, brain and muscle of rabbit, guinea pig and man were capable of hydrolysing potassium phenylsulphate and potassium *p*-cresylsulphate. The enzyme appears to be common to most animal tissues. In man arylsulphatase activity has been found in a large variety of tissues (Rosenfeld, 1925; Russo, 1947; Huggins & Smith, 1947), and various organs of the pig, calf (Hommerberg, 1931), dog, chicken (Morimoto, 1937), cat (Torda, 1943) and rat (Huggins & Smith, 1947) are also active.

It appears that the arylsulphatase activity of animal tissues is confined to substrates in which sulphuric acid is conjugated with hydroxyl groups of phenolic character, but the widespread distribution of the enzyme and its simultaneous occurrence with phosphatases and esterases (Hommerberg, 1931) have occasioned some doubt concerning the separate identity of the enzyme. Furthermore, the liver, muscle and kidney of horses and rabbits possess a myrosulphatase which releases inorganic sulphate from potassium myronate and, with the evidence available, it is not possible to decide the identity of myrosulphatase and arylsulphatase in animal tissues. No mammalian sources of gluco- or chondro-sulphatases have been reported. Tanaka (1938) has differentiated the phosphatase and arylsulphatase activities of rabbit tissues, but the lack of further work on the purification of the enzyme leaves its specificity ill defined.

Most investigations on mammalian arylsulphatase have been of a qualitative nature, and it is only recently that the introduction of sensitive colorimetric assay methods has enabled quantitative measurements of the enzyme to be made (Huggins & Smith, 1947; Robinson, Smith & Williams, 1951). In the rat considerable activity has been found in the liver and suprarenals whilst spleen and kidney are less potent. Decreased enzyme activity was shown in the liver after carbon tetrachloride damage, accompanied by a rise in serum arylsulphatase (Abbott & East, 1949), and certain tumours have been reported to have greater activity than their respective tissues of origin (Huggins & Smith, 1947). The methods employed by the above authors for rat tissues have been criticized (Dodgson & Spencer, 1953) on the grounds that tissue extracts, containing only a portion of the total enzyme, were used. To measure the total arylsulphatase present whole homogenates of the tissues must be used, but recovery of the liberated phenols (*p*-nitrophenol and 4-nitrocatechol), which are measured colorimetrically as an estimate of enzyme activity, is incomplete from such homogenates due to the action of reductases and other enzymes present.

Dodgson & Spencer (1953) have shown that the use of *p*-acetylphenylsulphate provides a reliable method for estimating arylsulphatase activity in whole homogenates of rat tissues and the development of an assay method using this substrate is presented in this paper.

## EXPERIMENTAL AND RESULTS

*Estimation procedure.* Rats and materials have been previously described (Dodgson & Spencer, 1953).

Whole homogenates of rat liver were used in establishing optimum conditions for the enzyme. The rats were killed by a blow on the back of the head and the livers rapidly removed and dropped into ice-cold water. Small portions were homogenized in a glass homogenizer (Potter & Elvehjem, 1936) for 2 min. with ice-cold buffer and were suitably diluted.

The ice-cold enzyme solution (0·6 ml.), adjusted to the required pH, was pipetted into 15 ml. tapered centrifuge tubes and pre-incubated for 4 min. before addition of 0·6 ml. substrate solution (potassium *p*-acetylphenylsulphate dissolved in buffer at the same pH as the enzyme solution). The mixtures (1·2 ml.) were incubated at 37·5° for the desired period and the enzyme action stopped and protein precipitated by addition of 4·8 ml. ethanol. After centrifuging, 1 ml. N-NaOH was added to 5 ml. of the clear supernatant and the absorption of the liberated *p*-hydroxyacetophenone was measured in the spectrophotometer at 327·5 mμ. against an ethanol blank. The estimations were duplicated and controls, containing the enzyme solution with the substrate added after incubation followed immediately by ethanol, were run simultaneously. It is unnecessary to include controls in which substrate is incubated with buffer since there is no hydrolysis of potassium *p*-acetylphenyl-

sulphate at 37·5° under the experimental conditions used. Although the specific absorption by protein at 327·5 mμ. is very slight, the ethanolic precipitation has been adhered to in order to clarify the incubation mixtures for the final measurement.

The amount of *p*-hydroxyacetophenone liberated in the incubation mixture is given by

$$\mu g.\ p\text{-hydroxyacetophenone} = (E_t - E_c) \times 43{\cdot}86,$$

where $E_t$ and $E_c$ are the observed extinctions of the test and control solutions respectively (Dodgson & Spencer, 1953). The percentage error involved in neglecting the decrease in absorption due to hydrolysis of the substrate is 0·35% which can be ignored.

*Optimum* pH. The wide range of pH optima reported for arylsulphatase can be attributed largely to the use of different sources of the enzyme by the various authors. In some cases the buffer has not been named and this point assumes importance since our results indicate that the buffer can greatly influence the optimum pH of rat-liver arylsulphatase. Takadiastase (Dodgson & Spencer, unpublished observation) is also affected in this way.

The only optimum pH recorded for rat tissues is that for the enzyme hydrolysing potassium *p*-nitrophenylsulphate. Abbott & East (1949) found optimum activity with this substrate at pH 6·6, whereas with the takadiastase enzyme the corresponding value was 6·12 (Huggins & Smith, 1947). Seligman, Chauncey & Nachlas (1951) have used potassium 6-bromo-2-naphthylsulphate as a substrate for rat-liver arylsulphatase at pH 5, and Robinson *et al.* (1951) used a pH of 6·0 with potassium 2-hydroxy-4-nitrophenylsulphate, but the authors do not state whether these values are those of the optimum pH for the enzyme.

Preliminary experiments with potassium *p*-acetylphenylsulphate suggested that the optimum pH of rat-liver arylsulphatase in the presence of 0·5 M-acetate was in the region 7·1–7·3 and that the optimum substrate concentration was 0·007 M. At pH 7·2 acetate has slight buffering action only, but measurements of pH before and after 1 hr. incubation of enzyme extracts with substrate in 0·5 M-acetate at pH 7·2 showed no significant difference. Water homogenates of most rat tissues, however, have a pH in the neighbourhood of 7·2 and possess a certain buffering power. It was decided to continue assays in the presence of acetate to allow comparison with work carried out simultaneously of the arylsulphatases of marine molluscs, bacteria and takadiastase (Dodgson, Lewis & Spencer, 1952; Dodgson & Spencer, unpublished). In 0·2 M-phosphate buffer the optimum activity of the enzyme of rat-liver homogenates was at pH 7·75.

Rat liver was homogenized in 0·5 M-sodium acetate and portions of the homogenate were adjusted to the required pH with 0·5 M-acetic acid and kept ice-cold until placed in the water bath. Substrate (0·007 M final concentration) was dissolved in 0·5 M-acetate at the desired pH. Measurements of pH before and after incubation (1 hr.) agreed to within 0·02 pH, within the limits 4·3–7·5. Above 7·5 the final pH was significantly lower than the initial value and in Fig. 1 points marked at a pH greater than 7·5 represent the pH midway between the initial and final values.

The pH-activity curve shown in Fig. 1 indicates that the arylsulphatase of rat liver has maximum activity at pH 7·2 under the experimental conditions specified.

*Optimum substrate concentration.* Variation of the substrate concentration affected enzyme activity in the manner expected from the Michaelis-Menten equations (see Fig. 2). With 0·5 M-acetate at pH 7·2 and 1 hr. incubation, optimal activity was shown at 0·007 M-potassium *p*-acetylphenylsulphate; greater concentrations of substrate showed no inhibitory effect. The plot of $[S]/V$ against $[S]$ was a straight

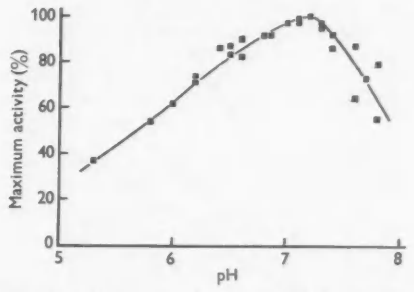


Fig. 1. pH-Activity curve for the arylsulphatase of rat-liver homogenates acting on potassium *p*-acetylphenylsulphate (0·007 M) in 0·5 M-acetate buffer at 37·5° during 1 hr.

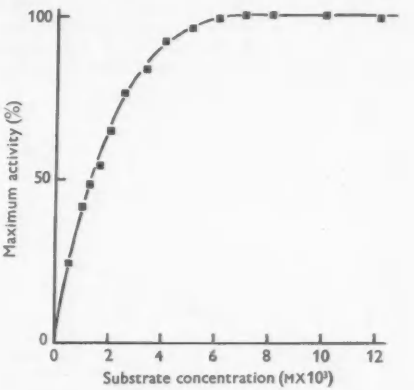


Fig. 2. Substrate concentration-activity curve for rat-liver arylsulphatase acting in 0·5 M-acetate, pH 7·2, on potassium *p*-acetylphenylsulphate at 37·5° during 1 hr.

line and the Michaelis-Menten constant, $K_m$, was calculated to be $1{\cdot}57 \times 10^{-3}$ M in a typical experiment. A second determination gave $K_m$ $1{\cdot}54 \times 10^{-3}$ M. No previous determination of $K_m$ of rat-liver arylsulphatase has been reported.

*Effect of time and enzyme concentration.* The effect of incubating rat-liver homogenates with the substrate for various periods was studied under the optimum conditions of pH and substrate concentration. The typical curves shown in Fig. 3 demonstrate that a linear relationship exists between

liberation of *p*-hydroxyacetophenone and time up to 1·25–1·5 hr. but some decrease in activity occurs after this point. Fig. 4 shows that under the optimum conditions the release of *p*-hydroxyacetophenone bears a rectilinear relationship to the enzyme concentration even when large amounts of sulphatase is possible; proteolytic destruction of the enzyme may also take place.

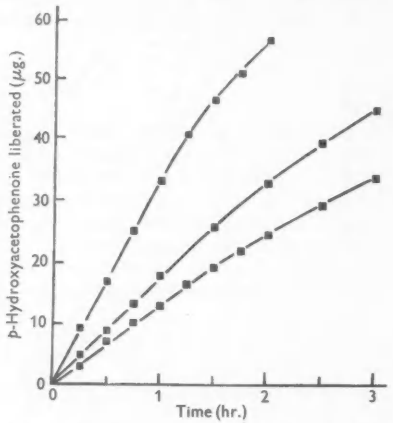


Fig. 3. Time-activity curves for rat-liver arylsulphatase acting at 37·5° on potassium *p*-acetylphenylsulphate in 0·5 M-acetate, pH 7·2. The results of three separate experiments, using different homogenates, are shown.

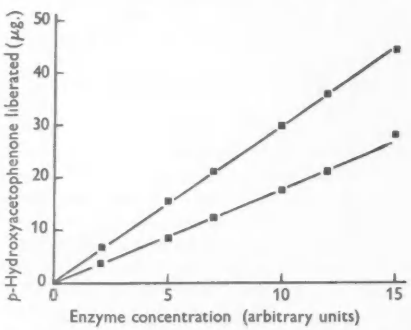


Fig. 4. Enzyme concentration-activity curves for rat-liver arylsulphatase acting on potassium *p*-acetylphenylsulphate (0·007 M) in 0·5 M-acetate at pH 7·2. Incubation was for 1 hr. at 37·5°. The results of two experiments, using different homogenates, are shown.

the phenol and inorganic sulphate are liberated. The decrease in activity of the enzyme with time often occurs when there is comparatively little hydrolysis and therefore it cannot be attributed to inhibition by the products of the reaction. In crude homogenates during incubation various enzymic reactions on natural substrates are taking place and the formation of products inhibitory to arylsulphatase is possible; proteolytic destruction of the enzyme may also take place.

*The effect of pre-incubation.* Since assay values were low when no precautions were taken to keep homogenates cold during manipulation prior to incubation with substrate, the loss of enzyme activity in crude homogenates incubated in the absence of substrate was investigated. Rat-liver homogenates in 0·5 M-acetate at pH 7·2 were incubated at 37·5° for various periods and subsequently assayed over 1 hr. with 0·007 M substrate. The results shown in Fig. 5 indicate that there is a large decrease in activity caused by pre-incubation at 37·5° and after 48 hr. all activity was lost. If enzyme solutions were kept ice-cold before incubation with substrate there was no loss of arylsulphatase activity even after 4–5 hr. but inactivation was still considerable at room temperature. For this reason it is essential to maintain all tissue preparations at 0° before assay.

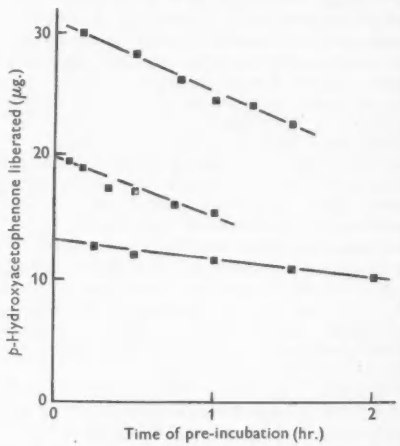


Fig. 5. The effect of pre-incubation at 37·5° on the arylsulphatase activity of rat-liver homogenates. The homogenate, in 0·5 M-acetate buffer, pH 7·2, was incubated at 37·5° for various periods and then assayed for arylsulphatase activity under the standard conditions. Three separate experiments, using different homogenates, are illustrated.

*The fractionation of arylsulphatase.* In the previous paper (Dodgson & Spencer, 1953) the recovery of various phenols from rat tissues was investigated with respect to their suitability for arylsulphatase assay. Preliminary work indicated that supernatant solutions of centrifuged homogenates of rat tissues prepared in a similar manner to those described by Huggins & Smith (1947) did not contain the full arylsulphatase present and, for this reason, it was considered that the use of whole

homogenates in the recovery experiments was necessary.

Further work confirms this point. Freshly removed rat liver was homogenized in ice-cold water or acetate buffer. Portions were centrifuged under ice-cold conditions for 15 min. at 2200 rev./min., the supernatant separated and the residue suspended in the same medium as that used in the original homogenization. The whole homogenate, supernatant and residue were then adjusted to pH 7·2 and made 0·5 M with respect to acetate, and the arylsulphatase activities assayed. The results presented in Table 1 demonstrate that it is necessary to use whole homogenates of liver in order to realize the full activity. Fractionation by centrifugation leads to loss of the enzyme and the supernatant solutions contain only 60–80% of the total activity. Fractionation of rat-liver arylsulphatase is being further studied.

Table 1. *The fractionation of rat-liver arylsulphatase by centrifugation of homogenates*

(The figures quoted are the mean values from three female rats.)

| Medium | pH | Activity (% of whole homogenate) Supernatant | Residue | Loss (%) |
|---|---|---|---|---|
| Water | — | 78·5 | 10·8 | 11·7 |
| 0·5 M-Acetate | 7·2 | 63·2 | 20·6 | 16·2 |

*Method of assay*

The preceding work established the optimum conditions for the arylsulphatase of rat-liver homogenates and a standard unit of activity may now be defined; one *p*-hydroxyacetophenone unit of arylsulphatase activity is that which liberates 1 $\mu$g. *p*-hydroxyacetophenone in 1 hr. from 0·007 M-potassium *p*-acetylphenylsulphate in the presence of 0·5 M-acetate, pH 7·2, at 37·5°.

The following procedure was adopted for assay work. The freshly killed adult rat was sectioned at the neck to allow free bleeding and the required organs dissected out and placed in ice-cold water. The organs were freed of fat, dried on filter paper, weighed and homogenized for 2 min. in ice-cold 0·5 M-acetate buffer, pH 7·2, using a glass homogenizer. Whole organs were used except in the case of intestine and liver where it was sufficient to use small samples. The tissue homogenates were then diluted with the buffer to suitable strengths so that the use of 0·6 ml. in the estimation procedure detailed earlier liberated 15–30 $\mu$g. *p*-hydroxyacetophenone. Owing to the fall in activity after 1·5 hr., 1 hr. incubations were adhered to. Ice-cold conditions were maintained for all solutions and apparatus throughout manipulation prior to incubation of the tissue with the substrate. With some tissues (brain, adrenals and ovaries) cloudiness develops in the final solution and further centrifugation is necessary before reading is made with the spectrophotometer.

Table 2. *The assay of arylsulphatase in liver samples from the liver* (7·7 *g.*) *of an adult female rat*

| Sample | *p*-Hydroxyacetophenone liberated by 0·6 ml. in 1 hr. ($\mu$g.) | *p*-Hydroxyacetophenone liberated/g. tissue in 1 hr. ($\mu$g.) | Error from mean (%) |
|---|---|---|---|
| 1 | 29·2 | 2040 | −1·92 |
| 2 | 31·8 | 2030 | −2·4 |
| 3 | 31·75 | 2090 | +0·48 |
| 4 | 16·32 | 2150 | +3·37 |
| 5 | 30·8 | 2150 | +3·37 |
| 6 | 27·7 | 2020 | −2·88 |

The use of small samples of liver rather than the whole organ was justified by showing that replicate samples from the same liver gave values which were within ±5% of the mean. The results of a typical experiment are given in Table 2.

A survey of the distribution of arylsulphatase in the tissues of rats has been made and the results are presented in Table 3.

## DISCUSSION

The distribution of the enzyme in the various tissues compares broadly with that found by Huggins & Smith (1947) but differs from the results of Robinson *et al.* (1951). The latter authors report that spleen

Table 3. *Arylsulphatase content of rat tissues*

(Expressed as *p*-hydroxyacetophenone units of arylsulphatase activity per g. of wet tissue together with the standard error calculated with $n-1$ for small samples. The number of determinations appears in parentheses. *P* is the probability calculated by the '*t*' test.)

| Organ | Male | Female | *P* |
|---|---|---|---|
| Liver | 3630±161 (21) | 2131±106 (26) | <0·01 |
| Adrenals | 1074±225 (6) | 940± 78 (7) | 0·40>0·30 |
| Kidney | 765± 36 (9) | 722± 43 (8) | 0·50>0·40 |
| Heart | 580± 42 (7) | 518± 40 (7) | 0·40>0·30 |
| Spleen | 550± 18 (9) | 536± 19 (6) | 0·70>0·60 |
| Lung | 450± 20 (8) | 442± 42 (8) | 0·90>0·80 |
| Brain | 203± 4 (5) | 184± 10 (7) | 0·20>0·10 |
| Muscle | 175± 10 (4) | 178± 12 (6) | 0·90>0·80 |
| Intestine | 166± 9 (4) | 142± 12 (6) | 0·30>0·20 |
| Testes | 432± 20 (8) | — | — |
| Ovaries | — | 403± 34 (7) | — |

is the most active organ, with liver only 10–20% as active, but since Robinson *et al.* and Huggins & Smith used tissue extracts and not whole homogenates and conditions of pH and substrate concentration that were optimal for the mould enzyme, their results are not strictly comparable with those of the present study. An added complication is that a strain difference appears to exist for arylsulphatase in rats (Dodgson & Spencer, unpublished). Although the numbers of samples in Table 3 are relatively small, the arylsulphatase activities of the livers were apparently normally distributed. The '$t$' test has therefore been applied and the results show that there is a significant difference between the enzyme activities of the male and female Medical Research Council hooded rats ($t = 8{\cdot}16$, $n = 45$, $P < 0{\cdot}01$). A similar difference has been observed for the liver alkaline phosphatase of Sprague-Dawley rats (Lowe & Salmon, 1951) but not for liver $\beta$-glucuronidase of mice (Levvy, Kerr & Campbell, 1948). Since the values in Table 3 are based on wet weights of liver it is possible that the difference in enzyme activity may be accounted for by a difference in water content of the livers of the two sexes. However, the ratio wt. of animal:wt. of liver is the same in males ($23{\cdot}3 \pm 0{\cdot}44$ (S.E.), $n = 35$) and females ($23{\cdot}3 \pm 0{\cdot}48$ (S.E.), $n = 37$). The other organs of the rat showed arylsulphatase activities which were similar for both sexes (Table 3). Further investigation of these observations is in progress.

The function of arylsulphatase in the animal body is as yet unknown. There is some indication that its action may be purely hydrolytic since, after injection into rats, the $^{35}$S-labelled ester sulphate group of sodium oestrone sulphate is recoverable in the urine to the extent of 75% as inorganic sulphate (Hanahan & Everett, 1950). On the other hand, sodium phenylsulphate can be quantitatively recovered unchanged from rabbit urine after oral administration (Garton & Williams, 1949). Alternatively, arylsulphatase activity may be the reverse process of what, in the intact animal, is normally a synthesizing action requiring a coupled energy-supplying system. Arylsulphate synthesis by rat tissues is known to be an endergonic reaction (DeMeio & Tkacz, 1952) which would not function under the conditions of assay employed in the present study.

In view of this possibility it is interesting to compare the distribution of the hydrolysing and the synthesizing action. The main points that emerge are that although the synthesizing ability of the liver and intestine are the same (DeMeio & Arnolt, 1944) the hydrolytic activities of these two organs are very different (Table 3) and that, whereas arylsulphatase appears to be common to all tissues, only liver, intestine and spleen were found to have synthesizing power (DeMeio & Arnolt, 1944). These differences do not necessarily show that different enzymes are responsible for synthesis and hydrolysis since there may be variations in other factors necessary for the synthesis of arylsulphates. Furthermore, the strain difference in rats used by DeMeio and in the present study must be taken into account since strain difference for both hydrolytic (Dodgson & Spencer, unpublished) and synthetic activities of rat tissues have been demonstrated (DeMeio & Arnolt, 1944).

## SUMMARY

1. The optimum conditions for the arylsulphatase of rat-liver homogenates have been established using potassium $p$-acetylphenylsulphate as the substrate. In 0·5 M-acetate the enzyme is optimally active at pH 7·2 and a substrate concentration of 0·007 M.

2. Arylsulphatase activity varied linearly with the concentration of the enzyme but declined after incubation with the substrate for periods longer than 1·25–1·5 hr. Incubation of rat-liver homogenates in 0·5 M-acetate at pH 7·2 in the absence of substrate caused considerable decrease in enzyme activity.

3. Fractionation of homogenates by centrifugation divided the enzyme between the supernatant and the residue but some loss of enzyme occurred.

4. A survey of the arylsulphatase content of rat tissues showed the greatest concentration in the liver. Adrenals, kidney, heart, spleen, lung, testes, ovaries, brain, muscle and intestine had lesser amounts, the values decreasing in the order given.

5. The arylsulphatase activity of the liver of male rats is significantly higher than that of females.

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# The Effect of Zinc Deficiency on the Aldolase Activity in the Leaves of Oats and Clover

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It is now well established that zinc is an essential nutrient element for plants. When the supply of zinc falls short of the physiological requirements of green plants, the retarded growth, the chlorosis, and the morphological changes in the organized structures which supervene, all suggest serious metabolic disturbances. Several biochemical lesions have been described. An abnormal protein metabolism (Bean, 1942), an interruption of tryptophan synthesis and thus of the production of β-indole-3-acetic acid (Tsui, 1948), an accumulation of inorganic phosphorus and an impairment of carbohydrate metabolism (Reed, 1946), have all been observed in zinc-deficient green plants.

In fungi, the retardation of growth in zinc-deficient media has been claimed to be due to a failure of carbohydrate metabolism, in particular, to a break of one or more links in the chain of anaerobic glycolysis (Foster & Denison, 1950). Marked changes in the enzymic constitution of zinc-deficient *Neurospora crassa* have been described by Nason, Kaplan & Colowick (1951).

The accumulation of reducing sugars and the relative shortage of sucrose and starch, which have been observed in the leaves of zinc-deficient tomato plants, imply an impairment of a fundamentally important step in carbohydrate metabolism which might possibly be attributed to a partial failure of aldolase activity (Reed, 1946). There seems little reason to assume, however, that zinc is concerned directly with the aldolase activity in the tissues of green plants, since the activity, *in vitro*, of aldolases from different sources is not invariably influenced by reagents which form unionized complexes with heavy metals. As there is no experimental evidence to support the suggestion (Reed, 1946) that the disordered carbohydrate metabolism in zinc-deficient plants may be attributable to a partial failure of aldolase, the aldolase activity has been estimated in the leaves of normal and of zinc-deficient oats (*Avena sativa* var. Algerian) and of subterranean clover (*Trifolium subterraneum* var. Bacchus Marsh); and the estimations have been extended to include a series of determinations of the aldolase activity in the tissues of oat plants grown in copper-deficient media.*

## EXPERIMENTAL

*The water cultures.* The main salts employed for the nutrient medium were freed from Zn and Cu by the exhaustive extraction of aqueous solutions at pH 7·0 with dithizone in $CCl_4$ and were subsequently recrystallized. The trace salts were purified by recrystallization.

The procedure adopted for growing the plants was briefly as follows: six plants were suspended through holes in the heavily paraffin-waxed wooden lids that covered each of the series of 3 l. Pyrex beakers containing the aerated culture solutions. Light was excluded from the solution by covering the outsides of the beakers with opaque paper. The solutions were aerated for 1 hr. each day with a stream of air, freed from dust, and saturated, by passing through a glass tower packed with glass Raschig rings immersed in glass-distilled water, and conveyed through plastic tubing. The water lost from the medium by transpiration and evaporation was replaced with glass-distilled water.

Each beaker held 3 l. of a culture solution containing: $KNO_3$, 1·0 g.; $KH_2PO_4$, 0·5 g.; $CaSO_4.2H_2O$, 0·5 g.; $MgSO_4.7H_2O$, 0·25 g.; ferric citrate, 0·04 g.; $MnSO_4.4H_2O$, 4 mg.; $Na_2MoO_4.2H_2O$, 0·25 mg.; $H_3BO_3$, 0·57 mg. (all/l.) Zinc sulphate and copper sulphate supplements were added to make the following final concentrations: for the zinc-deficient plants, 100 μg. Cu/l.; for the copper-deficient plants, 200 μg. Zn/l.; for the controls, 100 μg. Cu/l., 200 g.

* A brief note of the findings has been published (Quinlan-Watson, 1951).

Zn/l. The reaction of the media was approximately pH 5·0. To ensure that iron deficiency would not become a serious limiting factor, further additions of ferric citrate (5 ml. 0·8% ferric citrate/beaker) were made from time to time. The culture solutions were replaced after 50 days.

The oat and subterranean clover seeds from selected graded stocks were soaked overnight in glass-distilled water and then germinated on waxed cotton gauze suspended over glass-distilled water in a saturated atmosphere. The clover plants were transplanted to the water-cultures 10 days, and the oats 14 days, after germination. Plants in the Cu, Zn-supplemented medium grew normally. Deficiency lesions became apparent in the leaves of plants grown in the unsupplemented media within 50–60 days after transplanting. The oat plants were harvested 93–119 days, and the clover plants 141–146 days, after transplanting.

*Aldolase estimation.* Portions of green leaf showing no visible lesions, see below, were ground with a glass pestle and mortar to which were added 40 ml. of glass-distilled water per g. of green leaf. The suspension was strained through fine gossamer.

The aldolase activity of this suspension was estimated by the method of Sibley & Lehninger (1949), which depends upon the measurement of the colour density ($2 - \log T_{\lambda 540}$) of the 2:4-dinitrophenylhydrazine complex with the trioses formed by enzymic decomposition of hexosediphosphate, further modification of the triosephosphates being prevented by the addition of hydrazine. A portion of the suspension (0·1 ml.) was incubated at pH 8·6 (trihydroxymethylaminomethane buffer) with the substrate at 38°. After 30 min. the activity was suppressed by addition of trichloroacetic acid and the solutions, now each of total vol. 4·5 ml., were freed from turbidity by centrifuging. Trioses were estimated in 1 ml. portions of the clear supernatants.

The dry matter in the leaf suspension was ascertained by evaporating a 5 ml. portion and drying at 105°.

As the 2:4-dinitrophenylhydrazine complexes of the two trioses, glyceraldehyde and dihydroxyacetone, which are the products of aldolase activity, differ slightly in colour intensity, Sibley & Lehninger (1949) standardized the mixed colour against the alkali-labile phosphate of the resulting phosphotrioses. In the present study no attempt was made to standardize the spectrophotometric evaluation of the trioses, as the primary aim was to estimate the relative aldolase activities of normal and zinc-deficient plant tissues. However, in order to compare the activities ($I$) found in normal tissues with values reported in the literature, the unit of activity $Q/m$, employed here, was computed in the following manner.

The alkali-labile phosphorus, $P_{Alk}$, formed, is directly proportional to $2 - \log T_{\lambda 540}$ of the 2:4-dinitrophenylhydrazine complex (Sibley & Lehninger, 1949). Thus $P_{Alk} = m(2 - \log T_{\lambda 540})$ where $P_{Alk}$ is expressed in $\mu$g./ml. supernatant, and $m$ is a constant.

As 1 $\mu$mole of hexosediphosphate, HDP, is transformed by aldolase to 2 $\mu$moles of triosephosphates, i.e. to 62 $\mu$g. of $P_{Alk}$, $\frac{m(2 - \log T_{\lambda 540})}{62}$ is a measure of the number of $\mu$moles of HDP (per ml. supernatant) transformed in the reaction.

To convert to '$Q$' notation the final spectrophotometric reading of the trioses formed after 30 min. incubation of the leaf suspension with HDP under the defined conditions, the relative enzyme activities per hour per mg. of dry tissue are expressed by

$$Q_{HDP} = \frac{4{\cdot}5 \times 2 \times m(2 - \log T_{\lambda 540})\ 22{\cdot}4}{62 \times D}\ \mu\text{l. HDP/hr./mg.},$$

the factors 4·5 being the vol. of trichloroacetic acid supernatant and $D$ = mg. dry matter in the 0·1 ml. tissue suspension.

As '$m$', the constant, was not determined, activity of the preparations reported here are expressed as

$$\frac{Q}{m} = \frac{3{\cdot}25(2 - \log T_{\lambda 540})}{D}.$$

This provides a measure of the relative activities; the value of $m$, however, is probably between 6 and 7 when calculated from data supplied by Sibley & Lehninger (1949).

## RESULTS

### *The macroscopic lesions and symptoms exhibited by the deficient plants*

Obvious lesions characteristic of the deficiencies became apparent within 50–60 days of transplanting to the zinc-free and copper-free culture solutions.

*Avena sativa.* The copper-deficient oat plants displayed the white tips, the marginal necrosis, the dark bluish green colour of the leaves, and the marked tendency to tiller that have been observed and described by others (Rademacher, 1936; Riceman, Donald & Evans, 1940; Piper, 1942). Under the conditions imposed vegetative growth was relatively good, but the plants grown in the copper-deficient medium failed markedly to reach the height of their copper-supplemented controls, and they developed, in comparison, about twice the number of tillers.

The zinc-deficient oat plants were seriously dwarfed. Severe marginal necrosis of the leaves became evident, and yellowish-white spots surrounded by bronze-green, purple or brown areas were prevalent lesions. In some leaves these degenerative changes were so extensive that only the area immediately adjacent to the central vein remained green. The younger leaves, on emerging, were softer, paler green, and smaller than those of the zinc-supplemented controls. The material in which the aldolase activity was estimated was selected from the green parts of leaves in which necrosis of the distal portions, and a pale green colour, were the only lesions. Throughout the period of observation there was always a proportion of such leaves on each plant.

*Trifolium subterraneum.* The runner-internodes of the zinc-deficient clover plants failed to elongate and, because of this, and the fact that many more lateral runners developed, the plants had a closely bunched habit. The younger leaves were small, misshapen and, because of the failure of runner internodes to develop and of the excessive development of lateral runners, densely crowded. It is of interest, here, to recall that the failure of zinc-deficient tomato plants to elongate normally was attributed by Skoog (1940) to lack of auxin production, and that Tsui (1948), when investigating this phenomenon, showed that under these conditions the production of tryptophan, the probable precursor of the auxin, was materially reduced, and he concluded that zinc is involved in tryptophan production. Recently Nason (1950) and Nason *et al.* (1951) have demonstrated that the activity of the enzyme which catalyses the condensation of indole and

serine to form tryptophan is greatly decreased in the mycelium of *Neurospora crassa* grown in zinc-deficient media, and that although mycelial growth was stimulated by the addition of a complete mixture of amino-acids to the medium, the activity of this enzyme was not enhanced.

In addition to the striking alteration of the habit exhibited by the zinc-deficient subterranean clover plants, necrotic patches appeared on the older leaves and, in some cases, extended throughout the whole leaf.

### *Aldolase activity*

Only leaves or portions of leaves in which there were no obvious lesions were assayed for aldolase activity. The aldolase activities and some estimations of the zinc and copper contents of the leaves from the zinc- and the copper-deficient plants and of their supplemented controls are reported in Tables 1 and 2. From these figures it is obvious that zinc-deficiency, but not copper-deficiency, led to a considerable decrease of the aldolase activity in the leaves of both oat and subterranean clover plants.

Table 1. *Aldolase activity and* Zn + Cu *contents of oats* (Avena sativa *var. Algerian*) *cultured in* Zn- *or* Cu-*deficient media*

| Description | | μg./g. dry wt. of leaf Cu | Zn | Aldolase of leaf $Q/m$ |
|---|---|---|---|---|
| Control | 1 | 6·2 | 39·2 | 6·6 |
| | 5 | 4·4 | 28·8 | 8·1 |
| | 5 | 5·55 | 32·8 | 8·4 |
| | 9 | 5·35 | 23·0 | 5·4 |
| | 9 | 5·8 | 24·2 | 8·3 |
| | Av. | 5·46 | 29·6 | 7·4 |
| Cu-deficient | 4 | 1·4 | 39·2 | 7·3 |
| | 6 | 1·15 | 33·6 | 7·1 |
| | 6 | 1·15 | 30·0 | 7·0 |
| | 3 | 2·4 | 10·8 | 6·2 |
| | 3 | 2·1 | 10·2 | 6·0 |
| | Av. | 1·64 | 24·8 | 6·7 |
| Zn-deficient | 7 | 12·8 | 8·6 | 0·95 |
| | 8 | 9·5 | 8·2 | 1·3 |
| | 10* | 3·1* | 10·4* | 4·6* |
| | Av. | — | 9·1 | 2·3 |

* Both copper- and zinc-deficient.

Table 2. *Aldolase activity in leaf of subterranean clover* (Trifolium subterraneum *var. Bacchus Marsh*)

| Aldolase activity $[Q/m]$ of leaves | |
|---|---|
| Control | Zn-deficient |
| 2·4 | 0·42 |
| 2·2 | 0·28 |
| 1·9 | 0·32 |
| 2·2 | 0·34 |

## DISCUSSION

In the absence of an alternative channel of carbohydrate metabolism distinct from the customary course via the hexose- and triose-phosphates, any considerable decrease of aldolase activity might be expected to limit materially the rate at which the reversible transfer of phosphoglyceraldehyde to fructose-1:6-diphosphate may be accomplished, and so to impair seriously not only the production of hexoses during photosynthesis but also the exergonic glycolytic reactions. Thus it is not improbable that the reduction of the rate of carbohydrate metabolism by a partial failure of one of the intermediary enzymes is a biochemical lesion of fundamental importance in zinc-deficient green plants, for, in both species of plants studied, zinc deficiency led to a considerable decrease of the aldolase activity in the green leaves.

Three among a number of possible explanations of this phenomenon appear most plausible: zinc may be a constituent of the apo-enzyme, it may be directly or indirectly concerned with the synthesis of proteins or more specifically with the synthesis of aldolase itself, or it may function in association with, or as, a co-enzyme.

There is no direct evidence to support the first of these possibilities. The aldolase activity of yeast and of *Clostridium perfringens* has been reported to be inactivated by potassium cyanide, etc. Zinc, however, has no specific capacity to reverse these inhibitory effects; $Cu^{+}$, $Fe^{2+}$, $Co^{2+}$ share with $Zn^{2+}$ the capacity to reverse the inhibition of yeast aldolase by potassium cyanide, cysteine, pyrophosphate, and 2:2′-dipyridyl (Warburg & Christian, 1943), and similar inhibition of the aldolase of *Cl. perfringens* is reversed, apparently, only by $Fe^{2+}$ and $Co^{2+}$ (Bard & Gunsalus, 1950). Moreover, the aldolase of *Escherichia coli* has been reported to require $Mn^{2+}$ specifically for full activity (Knox, Stumpf, Green & Auerbach, 1948). Little is known as yet of the behaviour, *in vitro*, of the aldolases from the higher plants under these conditions. Aldolase activity is clearly widely distributed in green leaves (Tewfik & Stumpf, 1949), but partially purified aldolase from pea seeds (Stumpf, 1948) like the aldolases from rabbit muscle (Herbert, Gordon, Subrahmanyan & Green, 1940; Taylor, Green & Cori, 1948) or from rat muscle (Warburg & Christian, 1943) is apparently not activated by heavy metals.

The second possibility is contentious. It is not clear whether the retarded rate of synthesis of both protein and starch in incipiently zinc-deficient tomato plants, described by Bean (1942), is the result of a primary or of a secondary effect of the deficiency; and in view of the widely divergent effects of zinc deficiency on individual enzymes in *Neurospora crassa*—in the zinc-deficient mycelium of this organism the activities of the alcohol dehydrogenase and of a tryptophan-synthesizing enzyme are markedly decreased, the activity of aldolase and other enzymes are unchanged, and the

activity of diphosphopyridinenucleotidase is greatly increased (Nason *et al.* 1951)—there is no reason to attribute invariably the disorders of metabolism which occur in zinc deficiency to a primary impairment of protein synthesis. Copper deficiency has been claimed to have marked effects on the protein metabolism of green plants (Gilbert, Sell & Drosdoff, 1946; Wood & Womersley, 1946; Lucas, 1948; Gilbert, 1951), and so the observation that the aldolase activity in the leaves of copper-deficient oats remains unimpaired, suggests that the lowered aldolase activity in the leaves of zinc-deficient oats is not due primarily to a decreased protein synthesis; although zinc might be specifically involved in the synthesis of the enzyme itself.

The third possibility that the aldolases of green leaves are activated by $Zn^{2+}$ or by zinc-containing co-enzymes is being examined.

SUMMARY

Aldolase activity was found to be materially decreased in tissue suspensions prepared from the leaves of zinc-deficient plants of *Trifolium subterraneum* and of *Avena sativa*, but was unchanged by copper deficiency.

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# The Deoxyribonucleic Acid Content of the Rat Cell Nucleus and its Use in Expressing the Results of Tissue Analysis, with Particular Reference to the Composition of Liver Tissue

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In 1948, Boivin, Vendrely & Vendrely published figures for the deoxyribonucleic acid (DNA) content of the cell nucleus of vertebrates, based on the chemical analysis of a known number of isolated nuclei, and claimed that the amount of DNA per nucleus was constant for the somatic cells of different tissues and was approximately double that found in the haploid sperm cells. On the other hand, a considerable variation occurred between species, birds and fish having, in general, a lower DNA content per nucleus than mammals (Vendrely & Vendrely, 1948, 1949). The general trend of these

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observations was confirmed by Mirsky & Ris (1949) and for fowl tissues, by Davidson, Leslie, Smellie & Thomson (1951).

The original observations of the Vendrelys were apparently made on very small numbers of animals and did not include the rat. No systematic investigation of rat tissues appears to have been made apart from the few instances listed in the discussion below.

The results of Vendrely & Vendrely (1948, 1949) are average figures obtained by the gross chemical analysis of large numbers of isolated nuclei, but attempts have also been made to determine the DNA content of individual nuclei by quantitative photometric cytochemical methods employing the Feulgen reaction, methyl green staining or ultraviolet absorption (Ris & Mirsky, 1949; Pollister, 1950; Swift, 1950*a*, *b*; Kurnick, 1950; Pasteels & Lison, 1950 *a–c*; Mirsky & Ris, 1951; Pollister, Swift & Alfert, 1951; Frazer & Davidson, 1952). Such cytochemical methods give comparative figures rather than absolute amounts, but a comparison of the amounts of DNA in isolated nuclei as determined by chemical analysis and by photometric cytochemical analysis has been made by Leuchtenberger, Vendrely & Vendrely (1951) and by Leuchtenberger, Leuchtenberger, Vendrely & Vendrely (1952).

If the DNA content of the cell nucleus is indeed constant for the different somatic tissues of the same animal and for different individuals of the same species, the implications are far-reaching. They have already been discussed by Davidson & Leslie (1950 *a*, *b*), by Mirsky & Ris (1951) and by Davidson (1952). Since information concerning the composition of rat nuclei is limited and since the rat is so commonly employed in laboratory investigations, we have thought it desirable to examine the DNA content of rat cell nuclei from reasonably large numbers of animals under different conditions. We have, moreover, paid particular attention to the rat liver, which is so extensively used in biochemical work and in which the picture is complicated by the presence of polyploid and binucleate cells.

A brief preliminary account has already been published (Thomson, Heagy, Hutchison & Davidson, 1952).

## EXPERIMENTAL

*Animals.* Except where otherwise stated, the experiments were carried out on male albino rats (wt. 190–250 g.) from the departmental colony. Where indicated in the tables, male albino rats outside this weight range, female albino, and male and female hooded rats were also used.

*Diets.* The diets employed were as follows.

(*a*) A stock diet of 'rat cake' made by Levers Cattlefoods Ltd.

(*b*) A semi-synthetic diet similar to that used in the University of Illinois consisting of glucose, 73%; vitamin-free casein, 18%; salt mixture, 4%; arachis oil, 5%.

Each kg. of diet contained the following supplement: thiamine hydrochloride, 2·5 mg.; riboflavin, 5·0 mg.; nicotinic acid, 10·0 mg.; pyridoxin hydrochloride, 2·5 mg.; calcium pantothenate, 20·0 mg.; inositol, 100·0 mg.; *p*-aminobenzoic acid, 50·0 mg.; biotin, 0·1 mg.; folic acid, 1·0 mg.; 2-methylnaphthaquinone, 1·0 mg.; choline chloride, 1·0 g. Each animal received one drop weekly of a mixture of 1 g. α-tocopherol in 14 g. Radiostoleum (British Drug Houses Ltd.).

The salt mixture employed was that described by Griffin, Nye, Noda & Luck (1948).

(*c*) A protein-free diet similar to (*b*) above in which casein was replaced by glucose and additional phosphate was supplied as recommended by Kosterlitz (1947).

(*d*) A thiamine-deficient diet similar to (*b*) above but without thiamine hydrochloride.

(*e*) A thioacetamide-containing diet similar to (*b*) above but containing in addition 0·032% thioacetamide.

(*f*) A high-fat diet as described by Channon, Mills & Platt (1943) containing casein, 8%; beef fat, 40%; glucose, 46%; salt mixture, 5%; cod liver oil, 1%. Each rat received 10 μg. thiamine per day.

(*g*) A carcinogenic diet similar to that used by Griffin *et al.* (1948) containing casein, 18%; glucose, 73%; arachis oil, 5%; salt mixture, 4%; *p*-dimethylaminoazobenzene, 0·06%, and vitamin supplement. Control animals were kept for the same time on the same diet without the dimethylaminoazobenzene.

*Treatment of animals.* Some animals were subjected to the procedure of partial hepatectomy (removal of median and left lateral lobes), by the method of Higgins & Anderson (1931).

Others received alloxan as described by Diermeier, Di Stephano, Tepperman & Bass (1951). Such animals were fasted for 48 hr. before receiving, by subcutaneous injection, 175 mg. alloxan monohydrate/kg. body wt. Control animals received injections of saline. The development of diabetes was confirmed by blood-sugar estimations. Animals which did not develop diabetes were discarded.

*Isolation of nuclei.* All rats were killed by exsanguination under ether anaesthesia. The livers and other organs required for analysis were quickly excised, weighed and finely chopped with scissors. A small piece of tissue was taken for histological examination, about 0·5 g. was used for whole-tissue analysis, and the remainder used for isolation of nuclei by a modification of the citric acid procedure as employed by Mirsky & Pollister (1946). When the tissues could not be treated immediately they were preserved by freezing in solid $CO_2$.

The tissues were homogenized in a Waring, Atomix or Nelco Blendor, and without further use of the Blendor the nuclei were separated from the homogenates by differential centrifugation in a refrigerated centrifuge. The isolation procedure was checked microscopically as required. The number of nuclei in the final suspension was determined by counting in a haemocytometer chamber using 0·01 M-citric acid as diluting fluid. At least 1600 nuclei were counted by two observers working independently.

Leucocytes were isolated from the pooled citrated blood of about twelve rats by centrifuging in constricted centrifuge tubes according to the method used by Butler & Cushman (1940). They were suspended in 0·01 M-citric acid and counted in a haemocytometer in the usual way.

*Methods of analysis.* For whole-tissue analysis the method of Schmidt & Thannhauser (1945) as modified by Leslie & Davidson (1951) was followed. Phosphorus was determined

by the method of Allen (1940) in the lipid fraction, the DNA fraction and the ribonucleic acid (RNA) fraction. This last fraction contains not only nucleotide P derived from the breakdown of RNA but also small amounts of inorganic P derived from 'phosphoprotein' (Davidson, Frazer & Hutchison, 1951) and small amounts of non-nucleotide organic P (Smellie & Davidson, 1951; Davidson & Smellie, 1952). Figures for RNAP must therefore be regarded as approximate. In one experiment the individual nucleotides in the RNA fraction were determined by the paper-ionophoresis method of Davidson & Smellie (1952).

Protein N was determined by estimating the N content of a portion of the alkaline digest of the tissue residue from which acid-soluble and lipid material had been removed, and subtracting the calculated amount of nucleic acid N.

A portion of the nuclear suspension containing a known number of nuclei was treated with 0·5 vol. 30% (w/v) trichloroacetic acid (TCA) and submitted to the modified fractionation procedure of Schmidt & Thannhauser (1945). The precipitate of the fraction containing DNA and protein was washed with a few drops of ice-cold distilled water to remove TCA and was then dissolved in NaOH to a final concentration of 0·2 N. Portions of this solution were used for determination of (*a*) P by Allen's method, (*b*) deoxypentose by the diphenylamine method (Davidson & Waymouth, 1944) and (*c*) ultraviolet absorption calculated from the difference between the absorptions at 290 and 260 m$\mu$. as measured in a Beckman DU spectrophotometer or a Unicam SP500 quartz spectrophotometer. The diphenylamine and ultraviolet methods were calibrated with a standard prepared from thymus DNA. In this way three separate determinations were made of DNA based on phosphorus, deoxypentose and ultraviolet absorption. In all cases the results were expressed in terms of DNAP.

*Significance tests.* Student's '*t*' test was used to assess the significance of the difference between the mean values of two groups of data. When more than two groups were compared analysis of variance (Snedecor, 1946) was used. The conventional notation $P < 0{\cdot}05$ and $P < 0{\cdot}01$ is used below to indicate significance on the 5 and 1% levels respectively.

## RESULTS

*DNA content/nucleus in different tissues.* The mean values for the DNA content of the nuclei of various rat tissues are shown in Table 1 expressed in terms of picograms (pg.) DNAP/nucleus (1 pg. = $10^{-12}$ g.). The agreement between the three methods of estimation is reasonably good, although figures obtained by the deoxypentose estimation tend to be higher than those found by the other two methods.

The values obtained for the non-hepatic tissues are of the order of 0·65–0·70 pg. DNAP/nucleus (equivalent to 6·7–7·2 pg. DNA), although small intestine in both young and adult rats and salivary gland and pancreas in adult rats give slightly higher values. Analysis of variance, however, shows that no matter which of the three methods of estimating DNA is used there is no significant difference between the mean values found for the different non-hepatic tissues of either the young or the adult rat.

Liver nuclei from adult rats, on the other hand, give a value of the order of 0·9 pg. DNAP (equivalent to 9·3 pg. DNA). The corresponding figures for young rats and for embryos are, however, much lower and closer to those for the non-hepatic tissues.

*DNA content/nucleus in liver tissue.* Table 2, section 1, shows the effect of sex, strain, body weight and pregnancy on the DNAP content of the liver nuclei. The mean values for the different groups of animals fall very close together, and analysis of variance for each of the three methods of estimation does not indicate that the slight differences between them are significant.

The effect of various dietary treatments on the average DNAP content of the liver nuclei is shown in Table 2, section 2. Once again analysis of variance for each of the three methods of estimation does not indicate that the small differences between the means for the different groups of animals are significant.

The effect of a diabetogenic dose of alloxan is shown in Table 2, section 3. The values of '*t*' found for each of the three methods of estimation do not indicate a significant change in the average DNAP content of the liver nuclei although Diermeier *et al.* (1951) reported that, in the rat, alloxan caused a 12% increase in the DNA content of round liver nuclei of about 7 $\mu$. diameter.

*RNA content of isolated nuclei.* Figures for the average RNA content of the isolated nuclei are also presented in Tables 1 and 2. In Table 1 they are expressed as a range since only a few results were available for each tissue and these showed a wide scatter. The results obtained for liver nuclei were more numerous and consistent and are therefore expressed as mean values with standard errors. They must, however, be interpreted with considerable caution since, although nuclei are known to contain some RNA (McIndoe & Davidson, 1952), this constitutes only a small percentage of the total RNA of the tissue. The apparent RNA content of isolated nuclei will therefore be substantially increased if they are contaminated with cytoplasmic debris. Nevertheless, it is of interest to observe that fasting appears to cause a decrease in the average RNA content of the liver nuclei and that (in agreement with Laird, 1952) thioacetamide causes an increase.

*Composition of whole liver tissue.* The results of the analyses of whole liver tissue are shown in Table 3 expressed as (*a*) concentrations/100 g. fresh liver, (*b*) total amounts in mg./liver and (*c*) pg./pg. DNAP. Since the average DNAP content/nucleus in the liver of the adult rat is about 0·9 pg. irrespective of its sex, strain and body weight, and of the diet on which it has been maintained, the third method of expression gives an approximate estimate of the average cell composition. Similarly, the total

DNAP content of the liver in pg. gives an approximate estimate of the number of cells which it contains. Both these estimates are approximate unless corrections are made for the occurrence of binucleate cells in the liver and for the fact that a proportion of the liver substance is extracellular.

The usefulness of this method of expression may be appreciated from a consideration of the differences in liver composition between male and female rats of the same strain and of comparable body weight (Table 3, section 1). It will be seen that in both the albino and hooded strains there is little difference between the sexes in the concentrations of lipid phosphorus (LP), protein nitrogen (PN), or RNAP/100 g. liver. The concentration of DNAP, on the other hand, is about 20 % higher in females than in males and this difference is significant ($P < 0{\cdot}01$ in both strains). A similar difference has been observed by Lowe & Salmon (1951). The total amount of LP/liver is significantly lower in females than in males ($P < 0{\cdot}01$ for both strains) but the differences in the total amounts/liver of PN, RNAP and DNAP are of dubious significance and are not consistent between the two strains. When, however, the results are referred to DNAP it becomes clear that, in the female, the average mass of the cell and its average

Table 1. *Mean values for the nucleic acid content, in terms of deoxyribonucleic acid phosphorus (DNAP) and ribonucleic acid phosphorus (RNAP), of cell nuclei isolated from tissues of male albino rats on stock diet*

(Results are given ± S.E. The figures in brackets represent the number of observations. Adult rats weighed 195–250 g.; young rats weighed 35–90 g.)

| Tissue | Stage of development of animals | DNAP (pg./nucleus) as determined by: Phosphorus estimation | Deoxypentose estimation | Ultraviolet absorption | Range of RNAP (pg./nucleus) |
|---|---|---|---|---|---|
| Kidney | Adult | 0·652 ± 0·0202 (8) | 0·664 ± 0·0284 (7) | 0·663 ± 0·0145 (8) | 0·088–0·211 |
| | Young | 0·654 ± 0·0167 (3) | 0·702 ± 0·0440 (2) | 0·636 ± 0·0077 (3) | 0·055–0·139 |
| Spleen | Adult | 0·633 ± 0·0247 (8) | 0·662 ± 0·0454 (8) | 0·634 ± 0·0281 (8) | 0·057–0·156 |
| | Young | 0·685 ± 0·0278 (3) | 0·726 ± 0·0040 (2) | 0·694 ± 0·0455 (3) | 0·055–0·163 |
| Lung | Adult | 0·651 ± 0·0311 (6) | 0·692 ± 0·0388 (5) | 0·647 ± 0·0283 (6) | 0·031–0·262 |
| | Young | 0·595 (1) | — | 0·595 (1) | 0·067 |
| Small intestine | Adult | 0·738 ± 0·0175 (4) | 0·776 ± 0·0157 (4) | 0·684 ± 0·0250 (4) | 0·136–0·191 |
| | Young | 0·728 (1) | — | 0·712 (1) | 0·244 |
| Salivary gland | Adult | 0·733 ± 0·0170 (2) | — | 0·663 ± 0·0110 (2) | 0·106–0·258 |
| | Young | 0·637 (1) | — | 0·626 (1) | 0·208 |
| Leucocytes | Adult | 0·641 ± 0·0508 (4) | — | 0·661 (1) | — |
| Heart | Adult | 0·627 (1) | — | 0·689 (1) | — |
| Bone marrow | Adult | 0·670 (1) | — | — | — |
| Pancreas | Adult | 0·712 ± 0·0010 (2) | — | 0·726 ± 0·0050 (2) | — |
| Thymus | Young | 0·718 ± 0·0215 (4) | 0·719 ± 0·0450 (4) | 0·660 ± 0·0365 (4) | 0·050–0·127 |
| Liver | Adult | 0·913 ± 0·0115 (38) | 0·928 ± 0·0190 (30) | 0·870 ± 0·0157 (38) | 0·103–0·232 |
| | Young | 0·758 ± 0·0198 (13) | 0·788 ± 0·0267 (11) | 0·759 ± 0·0152 (13) | 0·134–0·251 |
| | Embryo | 0·780 ± 0·0717 (4) | 0·841 ± 0·0850 (4) | 0·721 ± 0·0763 (4) | 0·108 |

Table 2. *Mean values for the nucleic acid content, in terms of deoxyribonucleic acid phosphorus (DNAP) and ribonucleic acid phosphorus (RNAP), of rat liver nuclei*

(Results are given ± S.E. except where stated. The figures in brackets represent the number of observations.)

| Treatment of animal | Strain and sex | Body wt. (g. ± S.D.) Initial | Body wt. (g. ± S.D.) Final | DNAP (pg./nucleus) as determined by Phosphorus estimation | DNAP (pg./nucleus) as determined by Deoxypentose estimation | DNAP (pg./nucleus) as determined by Ultraviolet absorption | RNAP (pg./nucleus) |
|---|---|---|---|---|---|---|---|
| Section 1: | | | | | | | |
| All animals on stock diet (*a*) | Albino, M. | — | 221 ± 14 (43) | 0·913 ± 0·0115 (38) | 0·928 ± 0·0189 (30) | 0·870 ± 0·0157 (38) | 0·180 ± 0·00639 (23) |
| | Albino, M. | — | 271 ± 12 (14) | 0·873 ± 0·0287 (14) | 0·861 ± 0·0382 (11) | 0·844 ± 0·0294 (12) | 0·208 ± 0·0343 (5) |
| | Albino, F. | — | 199 ± 11 (14) | 0·942 ± 0·0397 (10) | 1·004 ± 0·0378 (6) | 0·912 ± 0·0557 (9) | 0·172 ± 0·0117 (9) |
| | Albino, F. (pregnant) | — | 234 ± 17 (12) | 0·893 ± 0·0222 (12) | 0·919 ± 0·0396 (10) | 0·832 ± 0·0275 (12) | 0·181 ± 0·0134 (3) |
| | Hooded, M. | — | 215 ± 12 (12) | 0·921 ± 0·0248 (12) | 0·965 ± 0·0371 (12) | 0·855 ± 0·0274 (12) | 0·182 ± 0·0148 (10) |
| | Hooded, M. | — | 291 ± 15 (13) | 0·903 ± 0·0316 (13) | 0·942 ± 0·0352 (13) | 0·851 ± 0·0287 (13) | 0·188 ± 0·0881 (12) |
| | Hooded, F. | — | 215 ± 13 (11) | 0·931 ± 0·0333 (10) | 0·951 ± 0·0606 (10) | 0·890 ± 0·0567 (10) | 0·169 ± 0·0850 (7) |
| Section 2. Albino rats of initial wt. 200–250 g. on various diets: | | | | | | | |
| Stock diet (*a*) | Albino, M. | — | 221 ± 14 (43) | 0·913 ± 0·0115 (38) | 0·928 ± 0·0189 (30) | 0·870 ± 0·0157 (38) | 0·180 ± 0·00639 (23) |
| Fast (72 hr.) after diet (*a*) | Albino, M. | 224 ± 12 (7) | 190 ± 11 (7) | 0·885 ± 0·0392 (6) | 1·026 ± 0·0210 (3) | 0·860 ± 0·0245 (5) | 0·144 ± 0·0134 (6) |
| High-fat diet for 14 days | Albino, M. | 223 ± 7 (17) | 198 ± 17 (17) | 0·889 ± 0·0229 (15) | 0·946 ± 0·0372 (10) | 0·871 ± 0·0293 (14) | 0·169 ± 0·0103 (11) |
| High-fat diet for 35 days | Albino, M. | 217 ± 6 (3) | 184 ± 11 (3) | 0·902 ± 0·0315 (3) | 0·937 ± 0·0547 (3) | 0·848 ± 0·0313 (3) | 0·160 ± 0·00658 (3) |
| Semi-synthetic diet (*b*) | Albino, M. | — | 223 ± 12 (16) | 0·893 ± 0·0294 (12) | 0·919 ± 0·0304 (11) | 0·852 ± 0·0279 (12) | 0·173 ± 0·0200 (6) |
| Fast (48 hr.) after diet (*b*) | Albino, M. | 219 ± 7 (14) | 205 ± 11 (14) | 0·916 ± 0·0191 (13) | 0·943 ± 0·0280 (10) | 0·883 ± 0·0244 (13) | 0·155 ± 0·0104 (7) |
| Protein-free diet (*c*) for 7 days | Albino, M. | 223 ± 6 (13) | 199 ± 10 (13) | 0·943 ± 0·0386 (9) | 0·990 ± 0·0628 (7) | 0·908 ± 0·0424 (9) | 0·197 ± 0·0152 (8) |
| Protein-free diet (*c*) for 15 days | Albino, M. | 217 ± 8 (12) | 180 ± 15 (12) | 0·872 ± 0·0308 (12) | 0·897 ± 0·0366 (11) | 0·837 ± 0·0320 (12) | 0·183 ± 0·0154 (8) |
| Thiamine-deficient diet (*d*) for 21 days | Albino, M. | 228 ± 20 (10) | 196 ± 17 (10) | 0·912 ± 0·0380 (7) | 0·917 ± 0·0594 (6) | 0·868 ± 0·0376 (7) | 0·141 ± 0·0119 (4) |
| Thioacetamide-containing diet (*e*) for 7 days | Albino, M. | 215 ± 12 (4) | 204 ± 18 (4) | 0·917 ± 0·0582 (4) | 0·934 ± 0·0748 (3) | 0·894 ± 0·0683 (4) | 0·263 ± 0·0314 (4) |
| Section 3: | | | | | | | |
| Alloxan-diabetic rats | Albino, M. | 216 ± 8 (5) | 187 ± 16 (5) | 0·891 ± 0·0125 (5) | 0·976 ± 0·0346 (4) | 0·888 ± 0·0134 (5) | 0·147 ± 0·00734 (5) |
| Controls for alloxan-diabetic rats | Albino, M. | 212 ± 2 (4) | 213 ± 6 (4) | 0·874 ± 0·0305 (4) | 0·886 ± 0·0365 (3) | 0·879 ± 0·0332 (4) | 0·147 ± 0·00805 (4) |

content of LP, PN and RNAP are 15–30 % lower than in the male. Similar calculations show that in pregnancy (Table 3, section 2) the total amount of DNAP (i.e. number of cells) in the liver is not significantly altered but the average cell mass increases by 12 % ($P<0.02$), the average cell content of LP by 20 % and of RNAP by 30 %. No significance can be attached to the increase in average cell content of PN. These results are in agreement with the finding of Campbell & Kosterlitz (1949) that in pregnancy there is a marked increase in the total RNA content of the liver.

Some figures for the composition of liver tissue from young animals and embryos are included in Table 3, section 2. The liver of the young animal has a composition not unlike that of the adult, but the embryo liver contains about twice as much DNAP and only half as much LP/100 g. fresh wt. Making allowance for the fact that the DNAP content/nucleus in this tissue is lower than in adult liver (0·75 pg. compared with 0·90 pg.) it would appear that the cells in the embryo liver are only half as large as those in the adult organ and that they contain only half as much RNAP and a quarter as much LP.

*Effects of various diets.* In considering the effects of the various dietary treatments shown in sections 3 and 4 of Table 3, it is convenient to consider separately their effect on the total number of cells in the liver and on the average cell composition. It is obvious that none of the treatments has had any marked effect on the total amount of DNAP/liver. The small differences between the means for the different groups have been found by analysis of variance to be non-significant. Therefore it appears that none of the treatments has significantly affected the total number of cells in the liver. The average cell composition, on the other hand, is drastically changed. On fasting after either the semi-synthetic or the stock diet, the average cell mass falls by about 35 % and the average cell content of LP, PN and RNAP by 20–30 %. In an experiment in which the nucleotides were separated from the RNA fraction by paper ionophoresis it was found that they accounted for 72·1 and 68·5 % of the phosphorus in this fraction in the livers of two fed rats and for 72·9 and 70·0 % in the livers of two rats fasted for 72 hr. It is clear from this observation that the amounts of nucleotides and of the other phosphorus-containing compounds in this fraction are about equally affected by fasting.

The thiamine-deficient diet produced results similar to those of fasting which may, however, be due not so much to the vitamin deficiency as to the resulting loss of appetite. The protein-free diet caused a 20 % fall in the average cell mass and a 30 % fall in the average cell content of LP, PN and RNAP during the first 7 days. During the second 7 days there was a further significant loss of LP, PN and cell mass ($P<0.01$, $P<0.02$, $P<0.01$ respectively) but the RNAP content did not decline further. The effects of the high-fat diet on the LP, PN and RNAP content of the cell were similar to those of protein deficiency, but less severe. This might be expected since the high-fat diet contains only 8 % protein. The average cell mass did not fall, and it is presumed that increased deposition of neutral fat balanced the loss of other cytoplasmic constituents.

Although our histological observations agree with those of Rather (1951), who found that thioacetamide increases the volume of the liver nuclei, this substance does not appear to have any effect on the composition of whole liver tissue. Similar findings have been recorded by Laird (1952).

Finally, the animals on the stock diet differed from those on the semi-synthetic diet in having a significantly higher content of LP/cell in the liver ($P<0.01$). The difference disappeared on fasting.

Administration of a diabetogenic dose of alloxan (Table 3, section 5) caused a significant fall in the weight of the liver ($P<0.01$) and in its total content of LP and RNAP ($P<0.01$ and $P<0.02$ respectively). Since, however, the falls in total content of DNAP (i.e. in number of cells/liver) and in average cell mass and content of LP and RNAP which accompanied these changes are not statistically significant, it is not possible to say whether the changes were due to a reduction of cell number or to a change in average cell mass and composition, or to both.

*Effects of carcinogenic diet.* The effects of the carcinogenic diet on the average DNAP content of the liver nuclei and the composition of whole liver are shown in Table 4. Compared with the controls, the animals which received the carcinogen had a lower average content of DNAP/nucleus and a higher concentration of DNAP/100 g. In those cases in which the total DNAP content of the liver was determined, it was generally found to be considerably increased. The total amounts and concentrations/100 g. of LP, PN and RNAP in the liver were not greatly affected but the ratios of these components to DNAP were markedly lower. Some animals developed tumours sufficiently large and well defined to be dissected out from the rest of the liver and analysed separately. Where this was done (rats no. H 5, 107–108, and 109–110) it was found that the tumour exhibited to an exaggerated degree the changes described above. The average DNAP content/nucleus was of the same order as the value found for the non-hepatic tissues of the normal animal. The ratios of LP, PN, RNAP and tissue mass to DNAP were only half as great as in the controls. In the non-tumour portion of the liver the average DNAP content/nucleus was also very low but the ratios of LP, PN, RNAP and tissue mass to

Table 3. *Mean composition of rat liver tissue in terms of lipid phosphorus (LP), protein nitrogen (PN), ribo*

(Results are given ± S.E. except where stated. The figur

| Treatment of animal | Strain and sex | Body wt. (g. ± S.D.) Initial | Body wt. (g. ± S.D.) Final | Liver wt. (g.) | LP (mg./100 g. liver) | PN (mg./100 g. liver) | RNAP (mg./100 g. liver) | DNAP (mg./100 g. liver) | |
|---|---|---|---|---|---|---|---|---|---|
| Section 1. Stock diet (*a*) | Albino, M. | — | 212 ± 8 (24) | 7·67 ± 0·190 (23) | 132·8 ± 3·31 (23) | 2523 ± 64·1 (14) | 93·2 ± 1·55 (23) | 21·6 ± 0·664 (23) | 10 |
| | Hooded, M. | — | 215 ± 12 (12) | 7·28 ± 0·262 (12) | 142·6 ± 4·30 (11) | 2618 ± 55·7 (11) | 91·1 ± 1·89 (11) | 22·9 ± 0·884 (11) | 10 |
| | Albino, M. | — | 276 ± 10 (10) | 8·53 ± 0·212 (10) | 127·7 ± 3·56 (10) | 2401 ± 53·0 (6) | 90·3 ± 3·81 (10) | 21·0 ± 1·01 (10) | 10 |
| | Hooded, M. | — | 281 ± 9 (8) | 8·83 ± 0·401 (8) | 141·1 ± 4·70 (8) | 2545 ± 95·8 (8) | 94·0 ± 3·62 (8) | 22·2 ± 0·940 (8) | 12 |
| | Albino, F. | — | 207 ± 8 (8) | 6·55 ± 0·348 (8) | 123·9 ± 2·89 (8) | 2522 ± 81·3 (3) | 99·9 ± 2·80 (8) | 27·4 ± 0·479 (8) | 8 |
| | Hooded, F. | — | 215 ± 13 (11) | 6·96 ± 0·295 (11) | 125·5 ± 2·44 (11) | 2598 ± 76·5 (11) | 99·7 ± 2·98 (11) | 27·7 ± 1·26 (11) | 8 |
| Section 2. Stock diet (*a*) | Albino, F. | — | 199 ± 11 (14) | 6·59 ± 0·266 (14) | 127·1 ± 2·14 (14) | 2566 ± 55·1 (8) | 98·6 ± 1·71 (14) | 26·9 ± 0·406 (14) | 8 |
| | Albino, F. (pregnant) | — | 234 ± 17 (11) | 7·65 ± 0·258 (11) | 135·0 ± 3·80 (10) | 2531 ± 61·5 (8) | 114·9 ± 4·22 (10) | 24·4 ± 1·17 (10) | 10 |
| | Albino (embryo) | — | — | — | 60·48 ± 3·52 (4) | — | 89·9 ± 3·46 (4) | 45·9 ± 3·46 (4) | |
| | Albino, M. | — | 35–90 | — | 121·5 ± 3·09 (13) | 2358 ± 39·2 (6) | 100·0 ± 2·48 (13) | 25·6 ± 0·799 (13) | |
| Section 3. | | | | | | | | | |
| Stock diet (*a*) | Albino, M. | — | 221 ± 14 (43) | 7·94 ± 0·149 (43) | 131·4 ± 2·24 (41) | 2469 ± 47·7 (27) | 91·8 ± 1·32 (41) | 21·9 ± 0·479 (41) | 10 |
| Fast (72 hr.) after stock diet | Albino, M. | 224 ± 12 (7) | 190 ± 11 (7) | 4·89 ± 0·158 (7) | 141·4 ± 2·98 (7) | 2955 ± 4·95 (2) | 109·1 ± 4·86 (7) | 33·4 ± 1·64 (7) | 6 |
| High-fat diet for 14 days | Albino, M. | 223 ± 7 (17) | 198 ± 17 (17) | 9·01 ± 0·455 (17) | 89·0 ± 3·14 (17) | 1922 ± 74·5 (7) | 68·1 ± 2·18 (17) | 20·6 ± 0·731 (17) | 7 |
| High-fat diet for 35 days | Albino, M. | 217 ± 6 (3) | 184 ± 11 (3) | 8·40 ± 0·378 (3) | 92·1 ± 1·97 (3) | 1891 ± 94·0 (3) | 67·1 ± 2·38 (3) | 20·0 ± 1·43 (3) | 7 |
| Section 4. | | | | | | | | | |
| Semi-synthetic diet (*b*) | Albino, M. | — | 223 ± 12 (16) | 8·22 ± 0·178 (16) | 110·5 ± 2·62 (15) | 2380 ± 116·0 (9) | 91·9 ± 2·44 (15) | 21·1 ± 0·702 (15) | 9 |
| Fast (48 hr.) after diet (*b*) | Albino, M. | 219 ± 7 (14) | 205 ± 11 (14) | 5·53 ± 0·179 (14) | 139·8 ± 2·30 (13) | 2603 ± 82·6 (8) | 107·7 ± 2·33 (13) | 33·3 ± 0·899 (13) | 7 |
| Protein-free diet (*c*) for 7 days | Albino, M. | 223 ± 6 (13) | 199 ± 10 (13) | 6·30 ± 0·209 (13) | 96·8 ± 3·13 (12) | 2080 ± 104·1 (8) | 81·6 ± 2·37 (12) | 25·5 ± 0·650 (12) | 6 |
| Protein-free diet (*c*) for 15 days | Albino, M. | 217 ± 8 (12) | 180 ± 15 (12) | 5·69 ± 0·302 (12) | 101·5 ± 1·99 (12) | 1845 ± 82·7 (8) | 92·2 ± 1·46 (12) | 29·4 ± 0·745 (12) | 5 |
| Thiamine-deficient diet for 21 days | Albino, M. | 228 ± 20 (10) | 196 ± 17 (10) | 5·43 ± 0·207 (10) | 130·1 ± 6·33 (10) | 2945 ± 135·0 (5) | 98·0 ± 2·91 (10) | 33·9 ± 1·38 (10) | 7 |
| Thioacetamide-containing diet for 7 days | Albino, M. | 215 ± 12 (4) | 204 ± 18 (4) | 7·93 ± 0·688 (4) | 111·7 ± 1·68 (3) | 2048 ± 19·2 (2) | 89·3 ± 2·08 (3) | 22·5 ± 0·618 (3) | 9 |
| Section 5. | | | | | | | | | |
| Alloxan-diabetic rats | Albino, M. | 216 ± 8 (5) | 187 ± 16 (5) | 6·24 ± 0·300 (5) | 128·6 ± 4·38 (5) | — | 93·5 ± 4·91 (5) | 25·7 ± 0·975 (5) | 7 |
| Controls for alloxan-diabetic rats | Albino, M. | 212 ± 2 (4) | 213 ± 6 (4) | 7·60 ± 0·158 (4) | 119·3 ± 3·58 (4) | — | 86·2 ± 2·75 (4) | 23·2 ± 0·975 (4) | 9 |

*ribonucleic acid phosphorus (RNAP) and deoxyribonucleic acid phosphorus (DNAP)*

figures in brackets are the number of observations.)

| LP (mg./liver) | PN (mg./liver) | RNAP (mg./liver) | DNAP (mg./liver) | LP (pg./pg. DNAP) | PN (pg./pg. DNAP) | RNAP (pg./pg. DNAP) | Tissue mass (pg./pg. DNAP) |
|---|---|---|---|---|---|---|---|
| 10·21±0·278 (23) | 194±5·21 (14) | 7·19±0·197 (23) | 1·66±0·0534 (23) | 6·28±0·264 (23) | 120·3±4·93 (14) | 4·38±0·120 (23) | 4710±130 (23) |
| 10·29±0·415 (11) | 189±3·05 (11) | 6·55±0·218 (11) | 1·63±0·0645 (11) | 6·40±0·368 (11) | 116·3±6·25 (11) | 4·04±0·148 (11) | 4480±216 (11) |
| 10·91±0·459 (10) | 208±2·24 (6) | 7·69±0·338 (10) | 1·79±0·0872 (10) | 6·22±0·346 (10) | 112·5±4·97 (6) | 4·32±0·0885 (10) | 4880±272 (10) |
| 12·43±0·632 (8) | 224±9·86 (8) | 8·31±0·546 (8) | 1·96±0·120 (8) | 6·41±0·249 (8) | 115·8±5·79 (8) | 4·25±0·112 (8) | 4560±186 (8) |
| 8·06±0·318 (8) | 158±7·39 (3) | 6·56±0·439 (8) | 1·80±0·0980 (8) | 4·52±0·113 (8) | 91·7±5·97 (3) | 3·64±0·0880 (8) | 3650±61·9 (8) |
| 8·68±0·306 (11) | 180±9·25 (11) | 6·94±0·363 (11) | 1·93±0·116 (11) | 4·49±0·235 (11) | 92·2±4·51 (11) | 3·51±0·0805 (11) | 3670±145 (11) |
| 8·33±0·312 (14) | 159±8·26 (8) | 6·51±0·332 (14) | 1·77±0·0698 (14) | 4·73±0·109 (14) | 95·4±2·93 (8) | 3·67±0·0710 (14) | 3720±58·0 (14) |
| 10·24±0·372 (10) | 192±9·81 (8) | 8·69±0·276 (10) | 1·84±0·0708 (10) | 5·61±0·212 (10) | 106·8±4·90 (8) | 4·79±0·244 (10) | 4180±182 (10) |
| — | — | — | — | 1·33±0·083 (4) | — | 1·98±0·140 (4) | 2220±161 (4) |
| — | — | — | — | 4·79±0·146 (13) | 89·9±2·32 (6) | 3·93±0·0805 (13) | 3960±137 (13) |
| 10·51±0·213 (41) | 198±4·78 (27) | 7·35±0·160 (41) | 1·75±0·0458 (41) | 6·14±0·182 (41) | 116·0±3·57 (27) | 4·27±0·0925 (41) | 4660±93·0 (41) |
| 6·91±0·266 (7) | 150±8·49 (2) | 5·33±0·292 (7) | 1·63±0·0914 (7) | 4·30±0·246 (7) | 87·1±2·30 (2) | 3·28±0·0987 (7) | 3030±148 (7) |
| 7·94±0·404 (17) | 182±9·22 (7) | 6·01±0·206 (17) | 1·82±0·0816 (17) | 4·27±0·179 (17) | 95·4±4·86 (7) | 3·33±0·0839 (17) | 4960±176 (17) |
| 7·73±0·256 (3) | 158±9·24 (3) | 5·63±0·277 (3) | 1·67±0·109 (3) | 4·64±0·226 (3) | 95·0±2·38 (3) | 3·37±0·124 (3) | 5060±358 (3) |
| 9·00±0·229 (15) | 193±8·70 (9) | 7·53±0·297 (15) | 1·73±0·0762 (15) | 5·26±0·157 (15) | 116·4±7·80 (9) | 4·38±0·0890 (15) | 4810±160 (15) |
| 7·68±0·200 (13) | 146±6·30 (8) | 5·93±0·207 (13) | 1·83±0·0550 (13) | 4·22±0·100 (13) | 79·2±3·02 (8) | 3·25±0·0631 (13) | 3030±84·4 (13) |
| 6·07±0·228 (12) | 133±7·89 (8) | 5·11±0·143 (12) | 1·60±0·0600 (12) | 3·79±0·0545 (12) | 81·9±6·40 (8) | 3·20±0·0488 (12) | 3950±102 (12) |
| 5·77±0·323 (12) | 108±7·21 (8) | 5·22±0·247 (12) | 1·67±0·0909 (12) | 3·46±0·0845 (12) | 62·3±1·80 (8) | 3·16±0·0894 (12) | 3430±90·2 (8) |
| 7·02±0·337 (10) | 153±10·80 (5) | 5·31±0·212 (10) | 1·83±0·0616 (10) | 3·86±0·154 (10) | 83·4±8·07 (5) | 2·93±0·124 (10) | 2990±118 (10) |
| 9·33±0·735 (3) | 183±20·5 (2) | 7·44±0·480 (3) | 1·88±0·1560 (3) | 4·98±0·179 (3) | 90·3±4·65 (2) | 3·98±0·107 (3) | 4460±120 (3) |
| 7·98±0·238 (5) | — | 5·79±0·206 (5) | 1·60±0·0559 (5) | 5·01±0·166 (5) | — | 3·62±0·0666 (5) | 3910±146 (5) |
| 9·05±0·161 (4) | — | 6·54±0·176 (4) | 1·76±0·0690 (4) | 5·16±0·170 (4) | — | 3·73±0·0610 (4) | 4340±182 (4) |

Table 4. *Mean composition of whole tissue and nuclei, in terms of lipid phosphorus (LP), pro phosphorus (DNAP), in the livers of rats fed a d*

(Control animals were kept for the same time on the same diet without the dimethylaminoazobenzene. Except wh

| Treatment and description of animals | Rat number | Body wt. at death (g.) | Liver wt. (g.) | Composition of whole liver tissue: LP (mg./100 g. liver) | PN (mg./100 g. liver) | RNAP (mg./100 g. liver) | DNAP (mg./100 g. liver) | LP (mg./liver) | PN (mg./liver) | RNAP (mg./liver) | DNAP (mg./liver) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Carcinogenic diet for 5 months. Initial body wt. 150–170 g.* | H 1 | 132 | 7·2 | — | 1950 | 70·6 | 32·9 | — | 140 | 5·08 | 2·37 |
| | H 2 | 136 | 7·8 | — | 2200 L | 75·0 L | 34·8 L | — | — | — | — |
| | H 3 | 105 | 6·2 | — | 2000 T | 79·4 T | 36·0 T | — | — | — | — |
| | H 5 | 147 | 13·6 | — | — | 78·8 L | 29·2 L | — | — | — | — |
| | | | | — | — | 53·8 T | 39·2 T | — | — | — | — |
| | H 6 | 97 | 3·3 | — | 2340 | 79·0 | 37·5 | — | 77 | 2·60 | 1·24 |
| | H 7 | 183 | 8·4 | — | 1980 | 63·1 | 28·0 | — | 166 | 5·30 | 2·35 |
| | H 8 | 139 | 6·8 | — | 1990 | 65·0 | 32·0 | — | 135 | 4·42 | 2·18 |
| | H 9 | 172 | 7·5 | — | 1970 | 68·7 | 38·5 | — | 148 | 5·15 | 2·88 |
| | H 10 | 170 | 9·0 | — | 2110 | 75·8 | 31·4 | — | 190 | 6·82 | 2·83 |
| | H 11 | 132 | 6·9 | — | 2170 | 75·0 | 34·0 | — | 150 | 5·18 | 2·35 |
| Controls for above: four animals of initial body wt. 163–195 g.* | — | 159–199 | 6·80 ± 0·791 | — | 2380 ± 91 | 83·3 ± 2·25 | 25·5 ± 0·851 | — | 161 ± 16·4 | 5·64 ± 0·592 | 1·73 ± 0·20 |
| Carcinogenic diet for 6 months. Initial body wt. 190–210 g. | 107 | 180 | 13·6 | 77·2 T | 1950 T | 72·7 T | 39·9 T | — | — | — | — |
| | 108 | 200 | 11·2 | 105·3 L | 2260 L | 76·0 L | 28·6 L | — | — | — | — |
| | 109 | 195 | 7·8 | 63·1 T | 1570 T | 68·8 T | 44·0 T | — | — | — | — |
| | 110 | 155 | 20·3 | 96·4 L | 2160 L | 64·8 L | 24·8 L | — | — | — | — |
| | 111 | 210 | 8·4 | 104·0 | 2350 | 78·6 | 27·3 | 8·74 | 197 | 6·60 | 2·29 |
| Controls for above: four animals of initial body wt. 190–210 g. | — | 200–243 | 6·53 ± 0·441 | 94·7 ± 3·83 | 2440 ± 38 | 78·6 ± 3·36 | 25·8 ± 0·477 | 6·15 ± 0·291 | 158 ± 8·1 | 5·10 ± 0·213 | 1·68 ± 0·11 |
| Carcinogenic diet for 2 months. Initial body wt. 190–210 g. | 465 | 149 | 9·2 | 97·0 | — | 81·7 | 37·0 | 8·92 | — | 7·52 | 3·40 |
| | 466 | 141 | 7·0 | 98·4 | — | 85·3 | 46·7 | 7·87 | — | 6·82 | 3·74 |
| | 467 | 139 | 6·2 | 109·5 | — | 83·3 | 37·3 | 6·82 | — | 5·16 | 2·31 |

T, tumour tissue only. L, residual liver tissue after removal of tumour nodules.

DNAP were closer to the normal values. Making allowance for the fact that the average DNAP content/nucleus was lower than in normal liver these observations might be interpreted as follows. The carcinogen caused an increase in the total number of cells in the liver of the order of 50–150 %. The new tumour cells were less than half the average size of the cells of the normal liver. They contained only 35 % as much LP, 35 % as much PN and 40 % as much RNAP.

*Effect of hepatectomy.* The results of the hepatectomy experiment are shown in Table 5. Throughout the 10 days following the operation the average DNAP content of the nuclei in the remaining lobes of the liver was consistently higher than the average value for unoperated animals of the same sex, strain and age (Table 2). The total amount of DNAP/liver rose steadily for the first 6 days, and during this period there were variations in the average DNAP content of the nuclei and in the ratios of LP and RNAP to DNAP in the whole tissue. From the sixth to the tenth day there was no further increase in the total amount of DNAP and the ratios of LP and RNAP to DNAP likewise showed little change.

## DISCUSSION

Our values for the average DNAP content/nucleus in normal adult rat liver (Table 1) are in fairly good agreement with those obtained by other workers using similar methods (Dounce, Tishkoff, Barnett & Freer, 1950; Ely & Ross, 1951*a*; Harrison, 1951; Leuchtenberger *et al.* 1951; Campbell & Kosterlitz, 1952; and Leuchtenberger *et al.* 1952). To calculate the DNA content/liver nucleus Price and his co-workers determined the amount of DNA/100 g. fresh liver and divided this figure by the number of nuclei/100 g., estimated by nuclear counts on a homogenate of a weighed portion of tissue (Price, Miller, Miller & Weber, 1950; Price & Laird, 1950). By this method they found somewhat greater values (10·0–14·0 pg. DNA, equivalent to 0·97–1·36 pg. DNAP). Rose & Schweigert (1952), using the same method, obtained even higher figures (14·9–15·4 pg. DNA, equivalent to 1·45–1·50 pg. DNAP). Although all DNA would be estimated by this method, any nuclei broken during homogenizing would not be counted and the estimated DNA/nucleus would therefore be too high. Thiis is a probable explanation of the high values obtained by these workers. On

*protein nitrogen (PN), ribonucleic acid phosphorus (RNAP) and deoxyribonucleic acid a diet containing* p-*dimethylaminoazobenzene*

(Except where otherwise indicated analyses and isolation of nuclei were carried out on portions of the whole liver.)

| Composition of whole liver tissue | | | | Composition of isolated nuclei | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | DNAP (pg./nucleus) as determined by | | | | |
| LP (pg./pg. DNAP) | PN (pg./pg. DNAP) | RNAP (pg./pg. DNAP) | Tissue mass (pg./pg. DNAP) | Phosphorus estimation | Deoxypentose estimation | Ultraviolet absorption | RNAP (pg./nucleus) | Remarks |
| — | 59·3 | 2·15 | 3040 | 0·802 | 0·916 | 0·774 | 0·138 | Diffuse tumour |
| — | 63·2 L | 2·16 L | 2870 L | 0·722 | 0·832 | 0·692 | 0·143 | |
| — | 55·5 T | 2·21 T | 2780 T | 0·792 | 0·859 | 0·750 | 0·133 | |
| — | — | 2·70 L | 3420 L | 0·792 | — | 0·744 | 0·192 | |
| — | — | 1·37 T | 2550 T | | | | | |
| — | 62·4 | 2·11 | 2670 | 0·823 | 1·165 | 0·824 | 0·121 | No obvious tumour |
| — | 70·7 | 2·25 | 3570 | 0·725 | 0·842 | 0·669 | 0·109 | |
| — | 62·2 | 2·03 | 3130 | 0·721 | 0·777 | 0·668 | 0·105 | |
| — | 51·2 | 1·78 | 2600 | 0·700 | 0·791 | 0·664 | 0·108 | Diffuse tumours |
| — | 67·2 | 2·41 | 3180 | 0·715 | 0·847 | 0·671 | 0·119 | |
| — | 63·8 | 2·21 | 2940 | 0·731 | 0·840 | 0·679 | 0·096 | |
| — | 93·8± 3·85 | 3·27± 0·082 | 3940± 126 | 0·951± 0·0122 | 0·974± 0·0435 | 0·907± 0·0499 | 0·190± 0·0151 | Results expressed as means ± S.E. |
| 1·93 T | 48·9 T | 1·82 T | 2510 T | 0·648 T | 0·643 T | 0·620 T | 0·302 T | |
| 3·67 L | 78·8 L | 2·66 L | 3500 L | 0·664 L | 0·735 L | 0·713 L | 0·294 L | |
| 1·43 T | 35·6 T | 1·56 T | 2270 T | 0·651 T | — | 0·574 T | 0·428 T | |
| 3·89 L | 87·1 L | 2·61 L | 4030 L | 0·687 L | 0·710 L | 0·677 L | 0·303 L | |
| 3·81 | 94·8 | 2·88 | 3660 | 0·729 | 0·823 | 0·718 | 0·255 | Liver almost normal in appearance |
| 3·68± 0·143 | 94·8± 5·21 | 3·05± 0·130 | 3880± 73 | 0·956± 0·0462 | 0·947± 0·0339 | 0·913± 0·0471 | 0·364± 0·0198 | Results expressed as means ± S.E. |
| 2·62 | — | 2·21 | 2700 | 0·735 | — | 0·738 | 0·136 | |
| 2·11 | — | 1·83 | 2140 | — | — | — | — | Diffuse tumours |
| 2·95 | — | 2·23 | 2680 | 0·732 | — | — | 0·136 | |

* Whole tissue analyses carried out by E. B. Smith and G. T. Mills.

the other hand, Cunningham, Griffin & Luck (1950), using their own modification of the citric acid method, found only 5·9–8·0 pg. DNA (equivalent to 0·57–0·78 pg. DNAP)/nucleus in the same organ.

Few results have been published for the nuclei of rat tissues other than liver. For normal intestine Ely & Ross (1951*b*) found that the average DNA content of the nuclei was 6·27 pg. (equivalent to 0·61 pg. DNAP). Cunningham *et al.* (1950) found the average DNA content of thymus nuclei to be 6·1–6·3 pg. (equivalent to 0·58–0·63 pg. DNAP). For kidney, Harrison (1951) and Leuchtenberger *et al.* (1951) found 5·7 and 5·5 pg. DNA (equivalent to 0·53 and 0·55 pg. DNAP) respectively. These observations are in reasonable agreement with those recorded in Table 1. However, in four experiments Mirsky and Kurnick found a value of 8 pg. DNA (equivalent to 0·78 pg. DNAP)/nucleus in both the kidney and liver of the rat (unpublished results quoted by Mirsky & Ris, 1951), and Kurnick (1951) later reported the DNAP content/kidney nucleus as 0·90 pg. (equivalent to 9·3 pg. DNA) for rats ranging in weight from 80 to 219 g.

The results in Table 1 show that with the exception of the liver the average DNA content/nucleus varies within very narrow limits in the rat tissues studied. Liver gives a figure about 30% higher than the other tissues. Ris & Mirsky (1949) and Leuchtenberger *et al.* (1951) have shown by photometric measurements on individual nuclei that the nuclei of the rat liver may be divided into three classes with respect to their content of DNA. Class I nuclei contain the same amount of DNA as the kidney nuclei. Classes II and III contain twice and four times this amount and it is suggested that they are tetraploid and octoploid nuclei. This is in agreement with the histological observations of Beams & King (1942), Sulkin (1943) and McKellar (1949), who found that a considerable proportion of the hepatocytes of rat liver are polyploid, as judged by the criterion of relative nuclear volume. Hence, the average DNAP content/nucleus in the liver depends on the relative proportions of diploid, tetraploid and octoploid nuclei. The lower figures found for the livers of young animals and embryos (Table 1) suggest that they contain a smaller proportion of polyploid nuclei, and McKellar's measurements of nuclear volume in young animals support this view. Swift (1950*a*), too, using the mouse, found by quantitative cytochemical methods

Table 5. *Mean deoxyribonucleic acid phosphorus (DNAP) content of the nuclei of rat liver regenerating after partial hepatectomy and the composition of whole liver tissue in terms of lipid phosphorus (LP), ribonucleic acid phosphorus (RNAP) and deoxyribonucleic acid phosphorus (DNAP)*

(Results given ± S.E. except where stated. Six animals for each time after operation.)

| Time after operation (days) | Body wt. (g. ± S.D.) | | Composition of whole liver tissue | | | DNAP (pg./nucleus) as determined by | | |
|---|---|---|---|---|---|---|---|---|
| | At operation | At death | Liver wt. (g.) | Total DNAP content/liver (mg.) | LP (pg./pg. DNAP) | RNAP (pg./pg. DNAP) | Phosphorus estimation | Deoxypentose estimation | Ultraviolet absorption |
| 1 | 225 ± 27 | 220 ± 31 | 3·02 ± 0·164 | 0·673 ± 0·0362 | 5·55 ± 0·204 | 4·48 ± 0·128 | 1·018* | 1·031* | 0·911* |
| 2 | 232 ± 12 | 218 ± 8 | 3·58 ± 0·114 | 0·769 ± 0·0310 | 5·91 ± 0·201 | 4·83 ± 0·208 | 1·258* | 1·355* | 1·217* |
| 3 | 227 ± 22 | 206 ± 24 | 3·92 ± 0·282 | 0·974 ± 0·0463 | 5·01 ± 0·224 | 4·08 ± 0·0783 | 1·022† | 0·985† | 1·060† |
| | | | | | | | 1·099† | 1·019† | 1·112† |
| 4 | 222 ± 35 | 202 ± 24 | 5·18 ± 0·364 | 1·224 ± 0·101 | 5·29 ± 0·110 | 4·72 ± 0·164 | 1·385† | 1·375† | 1·368† |
| | | | | | | | 1·151† | 1·243† | 1·138† |
| 6 | 231 ± 28 | 216 ± 17 | 5·42 ± 0·401 | 1·403 ± 0·0792 | 4·80 ± 0·184 | 4·46 ± 0·149 | 0·903† | 0·955† | 0·905† |
| | | | | | | | 0·975† | 1·015† | 0·968† |
| 8 | 260 ± 33 | 231 ± 23 | 5·12 ± 0·273 | 1·392 ± 0·0952 | 4·92 ± 0·326 | 4·61 ± 0·264 | 1·013† | 1·000† | 1·061† |
| | | | | | | | 0·949† | 0·983† | 1·016† |
| 10 | 228 ± 24 | 227 ± 12 | 6·15 ± 0·289 | 1·445 ± 0·0989 | 4·61 ± 0·250 | 4·25 ± 0·271 | 0·906‡ | 0·907‡ | 0·901‡ |
| | | | | | | | 1·014‡ | 1·059‡ | 1·064‡ |
| | | | | | | | 1·056‡ | 1·200‡ | 1·047‡ |

* Pooled tissue from six rats. † Pooled tissue from three rats. ‡ Pooled tissue from two rats.

that the proportion of class II and class III nuclei in the liver is lower in young animals than in adults.

Thus, in the somatic cells of the different tissues of a species, the average amount of DNA in the resting nucleus shows no significant variations in the absence of polyploidy. But in a heteroploid organ like the rat liver the average amount of DNA/nucleus is greater than the diploid value, due to the presence of tetraploid and octoploid nuclei containing two or four times the usual amount of DNA. This is consistent with the view that in the resting nucleus each set of chromosomes contains a constant amount of DNA.

In liver regenerating after partial hepatectomy the average DNAP content/nucleus during the first 4 days was 10–50 % above the mean value found for unoperated animals of the same sex, strain and body weight (Table 5, and Table 2, section 1). This observation is of particular interest because of the extremely high rate of growth and cell multiplication in this tissue (Higgins & Anderson, 1931; Brues, Drury & Brues, 1936; Abercrombie & Harkness, 1951). While mitosis occurs with a frequency of 1 in 7000 to 1 in 20 000 in the liver of a normal adult rat (Brues & Marble, 1937; Marshak & Byron, 1945) the frequency may reach 1 in 40 for parenchymal cells and 1 in 100 for bile-duct cells during the 4 days following partial hepatectomy (Abercrombie & Harkness, 1951). It is obvious that DNA must be synthesized during cell multiplication, but there have been conflicting reports on the relation of DNA synthesis to the stages of the mitotic cycle. According to Swift (1950*a*, *b*), DNA synthesis precedes mitosis, and nuclei that are about to enter mitosis contain a double complement of DNA. In a very rapidly growing tissue many nuclei will be preparing to divide and the average DNA content/nucleus will be increased if this view is correct. Our observations on regenerating liver support Swift's concept. On the other hand, Pasteels & Lison (1950*b*, *c*) concluded that half the normal nuclear content of DNA goes to each daughter nucleus at metaphase, and the full amount/nucleus is restored by synthesis of new DNA during telophase. Such a process would not affect the average DNA content/nucleus unless the nuclear membrane were restored before DNA synthesis were completed, in which case there would be a lower average DNA content/nucleus. Our observations of increased DNA/nucleus during regeneration would not appear to be compatible with the views of Pasteels & Lison (1950*b*, *c*).

Our observation that the average DNAP content/nucleus was higher on the second and fourth days after operation than on the other days would suggest that the mitotic rate reached peaks on the second and fourth days. Although we are inclined to attach importance to these changes observed

from day to day during the first 4 days after hepatectomy, more experimental data will be needed to evaluate their significance. Price & Laird (1950), using a different method of estimation, found that the average DNA/nucleus fell steadily from 18·2 pg. (equivalent to 1·77 pg. DNAP) 24 hr. after operation to 11·8 pg. (equivalent to 1·15 pg. DNAP) at 96 hr., and then remained fairly constant till the eighth day. In our experiments, and also in those of Price & Laird (1950), the average DNA content/nucleus was still above the normal level 6–10 days after operation, when the phase of rapid growth had ended. This may be due to a higher proportion of polyploid nuclei, since Beams & King (1942) and Sulkin (1943) found that the proportion of polyploid nuclei in the parenchymal cells is increased after partial hepatectomy. In this respect the difference between the normal and restored livers would be analogous to the difference between the livers of the young and the adult animals for, in both cases, cell multiplication leads to an increase in the proportion of polyploid nuclei. According to Beams & King (1942) these are formed during mitosis when the chromosomes from both nuclei of a binucleate cell unite on a common metaphase plate.

In summary, in the resting nucleus each set of chromosomes probably contains a constant amount of DNA. Most somatic tissues are diploid and their nuclei ordinarily have an amount of DNA corresponding to two sets of chromosomes. Some organs, such as the rat liver, normally have a greater average amount of DNA/nucleus due to the presence of polyploid cells. Before the onset of mitosis there is an increase in the DNA content of the nucleus, with the result that tissues that are growing very actively have a greater-than-normal average DNA content/nucleus, due to the presence of nuclei that are about to undergo mitosis.

In adult tissues in which mitoses are infrequent the average DNA content/nucleus should not be affected by any circumstance or treatment that does not lead to either multiplication or destruction of the nuclei. The results shown in Table 2, section 2, support this conclusion. None of the dietary conditions affected the average DNA content of the liver nuclei, although some of them produced marked changes in the chemical composition or histological appearance of the liver. Similar results for the effect of fasting have been reported by Mirsky & Ris (1951) and McIndoe & Davidson (1952), and for the effect of protein deficiency by Campbell & Kosterlitz (1952).

The response of young animals to protein deficiency may differ from that of adults. Ely & Ross (1951*a*, *b*) reported that in rats weighing 130–150 g. the average DNA content/nucleus in the liver, pancreas and small intestine was higher in animals maintained on a protein-free diet for 8–49 days than in animals maintained on a complete diet. Lecomte & Smul (1952) found by cytochemical estimations on individual nuclei that in rats weighing 60–65 g. protein deficiency for 28 days resulted in an increase in the DNA content of the liver nuclei. We did not attempt similar experiments because it is very difficult to evaluate the results. Many, possibly most, of the cells in the livers of young growing animals will eventually undergo mitosis. Before doing so they will double their content of DNA. It is conceivable that protein deficiency might inhibit growth and mitosis to a greater extent than it affects the premitotic synthesis of DNA, and this could result in an apparent increase in the average DNA content per nucleus.

The results for the composition of adult whole liver (Table 3) confirm and extend the observations made by Campbell & Kosterlitz (1950), by Mandel (1951) and by Davidson (1947, 1952) on the remarkable constancy of the total DNA content of the liver, i.e. of the total number of cells that it contains. In this organ the nuclei and their characteristic chemical component, DNA, form a constant element while LP, PN and cell mass (in short, the cytoplasm) vary a great deal, depending on the nutritional status of the animal. RNA follows an intermediate pattern; in the initial stages of conditions like starvation or protein deficiency there is a marked decrease in the RNA content of the liver, but this trend does not seem to be progressive like the changes in the constituents mentioned above. A similar trend can be seen in the data of Kosterlitz (1947). Such behaviour suggests that only part of the RNA in the cell is 'labile' and it might be related to the observations made by Muntwyler and his associates who found that protein deficiency had affected the RNA content of the supernatant fraction much less than that of other cellular fractions isolated from homogenized livers by differential centrifugation (Muntwyler, Seifter & Harkness, 1950; Seifter, Muntwyler & Harkness, 1950). The effect is more clearly seen if the data are re-calculated on the basis of total liver contents. Munro, Wikramanayake & Heagy (1952) have also observed that protein deficiency has a selective effect on the RNA content of different cellular fractions.

An important practical consequence of the constancy of the average DNA content is that it provides a fairly simple method of obtaining an estimate of the number of cells in a tissue for calculations of the average composition/cell. It is necessary to relate the results of tissue analysis to some characteristic that has been selected as a standard—most frequently wet or dry weight. This has the grave disadvantage that an apparent

change in the measured variable in fact may be due to a change in the standard or reference. For example, during a 48 hr. fast the concentrations of DNAP, LP and RNAP in terms of unit wt. of liver tissue increased (Table 3, section 4). But calculations of the total amounts/liver show that the DNAP content remained unchanged while the LP, PN and RNAP actually decreased by 20–30%. The apparent increase in these constituents, on a wet wt. basis, was due to a reduction of almost 40% in the liver wt. and could be quite misleading. Obviously, it is desirable that the standard of references should not change during the experiment.

The simplest solution of the problem is to quote the total amount of each component/liver, as is done in the example above. This has two limitations; it is only applicable when a whole organ is available for analysis and cannot be used for tissues such as bone marrow or biopsy specimens; changes in cell number cannot be distinguished from changes in average cell composition. A better procedure is to use the DNA content of the tissue as a measure of cell number and to use the ratios of other constituents to the DNA as measures of average cell composition. This overcomes the limitations of the first method and has the additional advantage that it may bring to light significant changes in cell composition that otherwise would be obscured by random variation in cell number. For example, the changes in the liver wt. and total content of LP and RNAP during the second week on the protein-free diet (Table 3, section 4) are not statistically significant while the change in total PN content is significant only on the 5% level. If the results are referred to DNAP, however, it becomes apparent that there are significant decreases in average cell mass ($P<0{\cdot}01$) and average cell content of LP ($P<0{\cdot}01$) and PN ($P<0{\cdot}02$) while the average RNAP content/cell has not changed.

We believe that when analytical results are expressed in relation to DNA content they suggest a more accurate interpretation than when presented in conventional form. It should be emphasized, however, that if the experiment results in a change in the average DNA content/nucleus this change must be taken into account in interpreting the results in order to visualize changes in the average composition/cell.

In our experiments on adult animals the average DNA content/nucleus changed in the hepatectomy experiment, which has already been discussed, and in the long-term experiment with a carcinogenic diet (Table 4) in which the average DNAP content/nucleus in the liver was much lower than the usual adult figure. In a tumour-bearing tissue at least three distinct processes might affect the average cell composition. (1) As the number of tumour cells increases the average cell composition for the tissue will approach the average composition of the tumour cells. If the tumour cells are smaller than the normal cells, the average cell mass for the whole organ will fall. If the tumour cells have diploid nuclei the average DNA content/nucleus will fall in an organ like the adult rat liver which normally contains polyploid nuclei (Table 4). If the tumour nuclei contain more DNA than the nuclei of the normal tissue, as happens in at least one fowl tumour (McIndoe & Davidson, 1952), the average will be higher in the cancerous tissue. (2) If the tumour is growing at a fairly rapid rate it may contain a significant proportion of nuclei that are about to divide and have more than the normal resting complement of DNA. This would tend to raise the average DNA content/nucleus. (3) If the growing nodules of tumour tissue compress the normal tissue to such an extent that it degenerates, the nuclei of the latter may undergo pyknosis and lose DNA (Leuchtenberger, 1950). This will reduce the average DNA content/nucleus. Of the three processes, the first usually would be much the most important quantitatively.

## SUMMARY

1. The average deoxyribonucleic acid phosphorus (DNAP) content/nucleus has been determined in various tissues of the rat by chemical analysis of isolated nuclei. For the liver the DNAP, ribonucleic acid phosphorus (RNAP), lipid phosphorus (LP) and protein nitrogen (PN) contents of the whole organ were estimated and the effects of such factors as sex, age, strain and diet were investigated. The average cell composition in the liver was calculated from the average DNAP content of its nuclei and the results of the whole tissue analysis.

2. In spleen, kidney, pancreas, small intestine, leucocytes, bone marrow, thymus, heart, lung and salivary gland the average DNAP content/nucleus is of the order of 0·65–0·70 pg. in both young and adult animals.

3. In the liver the average DNAP content/nucleus is of the order of 0·90 pg. in adult rats and 0·75–0·80 pg. in embryos and young animals.

4. In the adult rat the average DNAP content/nucleus in the liver does not vary with sex, strain or body wt. and is not affected by fasting, by a protein-free diet, by a thiamine-deficient diet, by a high-fat diet, by thioacetamide, by a diabetogenic dose of alloxan or by pregnancy.

5. The average DNAP content/nucleus is significantly decreased in the livers of adult rats following prolonged feeding of *p*-dimethylaminoazobenzene (DAB).

6. During the first 4 days following partial hepatectomy the average DNAP content/nucleus in the rat liver is 10–50% above the normal value.

7. The average cell mass and the average content/cell of RNAP, LP and PN in the liver are 20–30% lower in the normal female adult rat than in the male. In pregnancy the average cell mass and content/cell of RNAP, LP and PN are increased.

8. Fasting, protein deficiency and thiamine deficiency do not affect the number of cells in the liver but cause a decrease in the average mass and the RNAP, LP and PN content of its cells.

9. A diabetogenic dose of alloxan causes a decrease in liver wt. and in the total content of RNAP, LP and PN in the liver.

10. Thioacetamide has no effect on the composition of whole liver tissue, although it causes an increase in the size of the nuclei.

11. Prolonged administration of DAB results in an increase in the number of cells in the liver and a reduction of the average cell mass and average content of RNAP, LP and PN. These effects occur both in livers with obvious tumours and in livers that have not reached this stage.

12. The advantages of expressing results of tissue analyses in relation to DNA content are discussed.

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# The Isolation of L-Pipecolinic Acid from *Trifolium repens*

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The isolation of L-pipecolinic acid (piperidine-2-carboxylic acid) from leaves of *Trifolium repens* (white clover) has already been briefly reported (Morrison, 1952), and the present paper gives a detailed account of the isolation and identification of the substance.

During the examination by paper chromatography of the amino-acids extracted by aqueous ethanol from the dried foliage of *T. repens*, a distinctive unidentified ninhydrin-reacting substance was observed. This substance had an $R_F$ value in most solvents somewhat higher than that of proline, and produced with ninhydrin a characteristic deep violet-blue colour which faded very rapidly: Fig. 1 shows its position relative to the other amino-acids on a two-dimensional chromatogram of an ethanol extract of clover leaves. Unidentified spots, apparently similar, on paper chromatograms have been reported by Fowden (1951) from algae, by Campbell, Work & Mellanby (1951) from agenized wheat flour, and by Biserte & Scriban (1950, 1951) from malt and barley.

The substance was present in amount comparable with that of other amino-acids, and it seemed worthwhile to attempt to isolate and identify it. It was found possible to achieve a partial separation of the unknown from the other amino-acids by displacement chromatography on a column of a cation-exchange resin (Amberlite IR-100) after the method of Partridge & Westall (1949), and ultimately it was separated completely by partition chromatography on columns of powdered cellulose using a mixture of *n*-butanol with aqueous acetic or formic acid as solvent, and obtained in crystalline form. The progress of the separation was followed at each stage by means of paper chromatograms of the various fractions.

*Identification.* From its properties it appeared that the substance might be either pipecolinic acid (piperidine-2-carboxylic acid) or nipecotinic acid

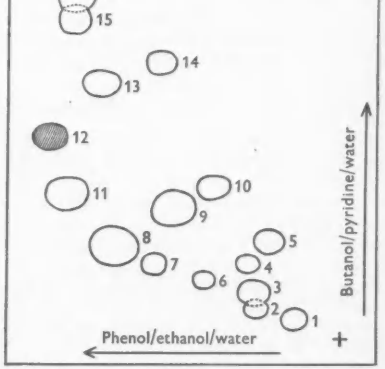


Fig. 1. Diagram of two-dimensional paper chromatogram of amino-acids in ethanol extract of leaves of *Trifolium repens* showing position of pipecolinic acid. 1, Aspartic acid; 2, glutamic acid; 3, asparagine; 4, glycine; 5, serine; 6, glutamine; 7, β-alanine; 8, γ-amino-*n*-butyric acid; 9, alanine; 10, threonine; 11, proline; 12, pipecolinic acid; 13, valine; 14, tyrosine; 15, phenylalanine; 16, leucine.

(piperidine-3-carboxylic acid). The described properties of optically active pipecolinic acid (Mende, 1896; Willstätter, 1901; Leithe, 1932) resembled closely those of the unknown substance. Nipecotinic acid has been described only in the racemic form (Ladenburg, 1892; Besthorn, 1895; Freudenberg, 1918), but its properties are very similar to

those of racemic pipecolinic acid (Ladenburg, 1891). Racemic specimens of both acids were prepared synthetically by the reduction of picolinic and nicotinic acids respectively with sodium in amyl alcohol (Clemo & Ramage, 1931), and their behaviour on paper chromatograms with four different solvents was compared with that of the natural preparation. In all cases, the unknown and pipecolinic acid behaved identically, and mixtures of the two were inseparable on chromatograms. Nipecotinic acid and the unknown had significantly different $R_F$ values.

This evidence, together with the optical activity, made it reasonably certain that the natural preparation was substantially L-pipecolinic acid, but since the value obtained for the specific rotatory power was lower than those given by Mende (1896) and Leithe (1932) for synthetic L-pipecolinic acid, the preparation was not directly comparable by classical methods with either pure L-pipecolinic acid or with the racemic acid. A specimen of the natural material was therefore racemized completely by heating with barium hydroxide, when the recrystallized product was found to be indistinguishable from the synthetic preparation. Additional confirmation of identity was provided by X-ray powder photographs and also by preparation and comparison of the hydantoins.

## EXPERIMENTAL

### *Identification of free amino-acids in leaves of* Trifolium repens

*Extraction.* A sample (5 g.) of clover leaves, oven-dried at 70° and ground in a mortar, was heated on the steam bath for 1 hr. with 70% (v/v) aqueous ethanol (50 ml.). The extract was filtered from the insoluble material which was washed with additional solvent. The combined extract and washings were evaporated almost to dryness under reduced pressure, diluted to 25 ml. with water, and extracted thrice with $CHCl_3$ (10 ml. each time). The aqueous solution was passed through a column of cation-exchange resin (Amberlite IR-100, 50–100 mesh/in., 1 × 10 cm.) which absorbed the amino-acids; these were then eluted with 0·5 N-$NH_3$. The eluate was concentrated to 5 ml., and the resulting solution examined by paper chromatography.

*Paper chromatography.* Whatman no. 1 filter paper (47 × 57 cm.) was used for two-dimensional paper chromatograms (Dent, 1948) with the following solvents: (1) a mixture of phenol, ethanol and water in the proportions 3:1:1 by volume, and (2) a mixture of *n*-butanol, pyridine and water in equal proportions by volume. The papers were dried at 80°, sprayed with ninhydrin (0·05%) in water-saturated butanol, and developed at 80° for 10 min.

Satisfactory chromatograms were obtained using 20–30 µl. of the concentrated plant extract.

The course of the separation of pipecolinic acid from the other amino-acids extracted from clover was followed by means of one-dimensional paper chromatograms of the fractions on Whatman no. 1 paper with the solvent phenol/ethanol/water (3:1:1 by volume).

### *Isolation of pipecolinic acid from leaves of* Trifolium repens

*Material.* Freshly cut leaves of white clover were dried at 20° for 48 hr., and then at 70° for 16 hr. The dry material was ground in a C and N Junior Mill (Christy and Norris Ltd., Broomfield Road Ironworks, Chelmsford).

*Extraction.* The material (500 g.) was extracted with 70% aqueous ethanol (4 l.) under reflux on the steam bath for 2 hr. The extract was filtered, and the insoluble material was again extracted with solvent (4 l.). The insoluble residue was washed with 70% ethanol (1 l.). The combined extracts and washings were evaporated under reduced pressure, and the residue was diluted with water to 4 l. and shaken up with benzene (500 ml.). The aqueous solution was partially freed from benzene in a separating funnel, and the process completed in a centrifugal separator.

To remove tyrosine and phenylalanine, the solution was treated with charcoal (10 g.) which had been pretreated with 5% (w/v) acetic acid (Partridge, 1949): after 2 hr. the solution was filtered, and the filtrate and washings concentrated to 1 l.

A column (2·0 × 60 cm.) of Amberlite IR-100 resin (50–100-mesh/in.) was prepared, and treated first with 2 N-HCl (500 ml.) and then with distilled water (2 l.). The clover extract was passed through the column at a rate of about 500 ml./hr., followed by distilled water (500 ml.). The effluent was found to be free from amino-acids, and these were displaced from the resin by 0·2 N-$NH_3$ which was passed through at a rate of about 100 ml./hr. until $NH_3$ appeared in the effluent. The solution was evaporated under reduced pressure, and the volume was adjusted to 500 ml.

*Fractionation of amino-acids by displacement chromatography.* A column of Amberlite IR-100 was prepared in two sections: the upper section (2·2 × 57 cm.) contained 100–200 mesh/in. resin, and the lower section (1·4 × 40 cm.) contained 100–200 mesh/in. resin. The column was pretreated with 2 N-HCl (500 ml.), and then with water (2 l.), and the amino-acid solution was allowed to run through at a rate of about 100 ml./hr. The effluent liquid was free from amino-acids. The latter were displaced from the column by $NH_3$ (0·15 N) which was passed through at a rate of about 25 ml./hr., and the effluent was collected in 6 ml. fractions by means of an automatic fraction collector (Hough, Jones & Wadman, 1949). Amino-acids were detected in 87 fractions, and these were examined by paper chromatography: aspartic acid, 1–10; serine, 4–19; threonine, 4–19; asparagine, 12–52; alanine, 17–47; glycine, 32–45; proline, 34–56; valine, 40–64; leucine, 48–74; pipecolinic acid, 51–72; γ-aminobutyric acid, 57–87; β-alanine, 58–72. Fractions 51–72 containing all the pipecolinic acid, together with most of the leucine, valine and β-alanine, about half of the γ-aminobutyric acid and some of the proline, were combined and evaporated to dryness under reduced pressure.

Another resin column was prepared, this time in three sections: 2·5 × 25, 1·5 × 10 and 1·0 × 5·5 cm. respectively, each section containing Amberlite IR-100 (100–200 mesh/in.). The column was pretreated with 2 N-HCl and washed with water as before, and fractions 51–72 from the first column, in 100 ml. of water, were run through followed by distilled water (500 ml.). The amino-acids were displaced with $NH_3$ (0·15 N) as before, and the effluent was collected in 6 ml. fractions. Amino-acids were found in 45 fractions and these were examined by paper chromatography: proline,

1–8; valine, 1–21; leucine, 7–34; pipecolinic acid, 5–45; γ-aminobutyric acid, 26–40; β-alanine, 25–30. Very little further separation was achieved on this column.

*Fractionation on columns of powdered cellulose.* A cellulose column (3·5 × 110 cm.) was prepared by pouring a slurry of Whatman cellulose powder in acetone into a glass tube (Campbell *et al.* 1951). When the cellulose had settled and compacted, the acetone was displaced by the upper phase of an equilibrated mixture of *n*-butanol, water and acetic acid (6:3:1 by vol.) similar to that described by Partridge (1948). Fractions 5–23 from the second resin column were combined, taken to dryness (6·30 g.) and dissolved in the smallest possible volume of the solvent along with 10 mg. of methyl orange to act as a marker. The solution was run on to the column and allowed to drain into the cellulose. The column was developed until the band of methyl orange reached the base and began to appear in the effluent which was then collected in 5 ml. fractions. A sample from each fraction was tested for the presence of amino-acids and examined by paper chromatography: methyl orange, 1–21; leucine, 46–75; valine, 92–145; pipecolinic acid, 99–178; proline, 199–230. Fractions 146–178 thus contained pipecolinic acid free from other amino-acids, and fractions 99–145 contained a mixture of pipecolinic acid and valine.

The *n*-butanol-acetic acid solvent was then displaced from the column by the lighter phase of an equilibrated mixture of *n*-butanol, water and formic acid (10:6:1 by vol.). Fractions 24–45 from the second resin fractionation and fractions 99–145 from the first cellulose fractionation were combined, dissolved in 15 ml. of this solvent along with 10 mg. of methyl orange, and run on to the column which was then developed with the solvent. The effluent was collected in 6 ml. fractions: methyl orange, 1–30; leucine, 170–231; valine, 180–259; pipecolinic acid, 221–359; γ-aminobutyric acid, 320–599. Here again, only a partial separation was achieved: fractions 221–259 contained pipecolinic acid with leucine and valine, and fractions 320–359 contained pipecolinic acid and γ-aminobutyric acid.

Another cellulose column (3·0 × 120 cm.) was prepared, this time using solka floc powdered sulphite pulp pretreated as described by Campbell *et al.* (1951). The cellulose was packed dry in small portions (Hough *et al.* 1949) and the tube was surrounded by an electrically heated water jacket maintained at about 35°. The cellulose was first wetted by acetone, fed in from the base, and the acetone was then displaced from above by the *n*-butanol-formic acid solvent already described. Fractions 221–259 and 320–359 from the second cellulose fractionation were combined, taken to dryness, and dissolved with 10 mg. of methyl orange in 10 ml. of the solvent: this solution was run on to the column followed by 10 ml. of the solvent. A constant-head reservoir of the solvent was placed in position, and collection of 2 ml. fractions was commenced when methyl orange appeared in the effluent. The fractions were examined for amino-acids by paper chromatography with the following results: methyl orange, 1–12; leucine, 126–133; valine, 246–266; pipecolinic acid, 293–397; γ-aminobutyric acid, 542–797. On this column a complete separation was thus obtained of all the remaining amino-acids.

*Crystallization.* Fractions 146–178 from the first cellulose column, fractions 260–319 from the second, and fractions 293–397 from the third were combined, and the solvent removed under reduced pressure. The residue in aqueous solution was decolorized with charcoal (0·1 g.), taken to dryness (0·632 g.) and recrystallized from 95% (v/v) ethanol by dilution with ether and cooling to 5°. The colourless microcrystals (0·510 g.) were washed with ether and dried in a vacuum desiccator.

### *Identification of the natural amino-acid as* L-*pipecolinic acid*

*Properties.* The natural preparation was very soluble in water, fairly soluble in anhydrous methanol, slightly soluble in anhydrous ethanol, and practically insoluble in acetone or ether. It was difficult to induce crystallization from solvents containing water, and it was found that the substance could most readily be crystallized from a mixture of anhydrous methanol and acetone. The reaction of the aqueous solution was neutral. The amino-acid formed an intense violet-blue coloured copper salt, soluble in ethanol, and readily prepared by heating in 95% ethanol with copper carbonate. The specific optical rotation (measured in a 0·5 dm. microtube) was: $[\alpha]_D^{18°}$ – 25·4° in water (*c*, 5). The m.p. (microstage) was 271° (decomp.). A small quantity was sublimed without obvious decomposition at 180–200°/0·5 mm. Hg pressure, and obtained in the form of fine fibrous needles, m.p. (microstage) 260° (decomp.). The following analytical figures were obtained (Weiler & Strauss). (Found: C, 54·8; H, 8·5; N, 10·2. Calc. for $C_6H_{11}O_2N$: C, 55·8; H, 8·5; N, 10·9%. Primary amino N (Van Slyke, 1929), nil.)

*Preparation of synthetic* DL-*pipecolinic acid.* Picolinic acid (2 g.) was reduced by Na in amyl alcohol (Clemo & Ramage, 1931). The crude pipecolinic acid hydrochloride was converted to the free acid and purified by absorption on a column of Amberlite IR-100 cation-exchange resin and fractional displacement with $NH_3$ (0·15 N). Forty-two 5 ml. fractions containing amino-acid were collected and tested for homogeneity by paper chromatography: all showed a single spot ($R_F$ 0·81 in phenol/ethanol/water 3:1:1) except the last two which showed in addition a spot with $R_F$ 0·59. Fractions 1–40 were combined, treated with charcoal, and taken to dryness. The residue (1·463 g.; 70%) was recrystallized from a small volume of anhydrous methanol by diluting with acetone, m.p. (microstage) 244° (decomp.). A specimen sublimed at 200°/0·5 mm. Hg pressure had m.p. 236° (decomp.). (Found: N (Kjeldahl), 10·8%.)

DL-*Nipecotinic acid.* This was prepared from nicotinic acid by a method similar to that used for DL-pipecolinic acid. The recrystallized product had m.p. (microstage) 250° (decomp.).

*Chromatographic behaviour.* The behaviour of the natural amino-acid on one-dimensional paper chromatograms was compared with that of synthetic pipecolinic and nipecotinic acids using the following solvents: (*A*) phenol/ethanol/water (3:1:1), (*B*) *n*-butanol/pyridine/water (1:1:1), (*C*) *n*-butanol/water/acetic acid (6:3:1), (*D*) *n*-butanol/water/formic acid (10:6:1) (proportions by vol.). The $R_F$ values obtained for the three preparations, together with those for proline, are shown in Table 1. It was found that a mixture of the natural amino-acid and synthetic pipecolinic acid could not be separated in any of the four solvents, and invariably produced a single spot.

The coloured products produced with ninhydrin by the three preparations were very similar in appearance: in daylight, the colour of the spots was usually less reddish than the usual amino-acid spot and faded much more rapidly; but the colour in ultraviolet light was most distinctive—a bright cherry-red in contrast to the dark blue produced with most amino-acids. In equal concentrations the natural amino-

acid and synthetic pipecolinic acid produced spots of equal intensity, but the same concentration of nipecotinic acid produced a much weaker spot.

Table 1. *$R_F$ values of pipecolinic acid, nipecotinic acid and proline*

| Solvents ... ... ... | $R_F$ *A* | *B* | *C* | *D* |
|---|---|---|---|---|
| Natural pipecolinic acid } Synthetic pipecolinic acid } | 0·81 | 0·44 | 0·24 | 0·36 |
| Nipecotinic acid | 0·78 | 0·30 | 0·17 | 0·25 |
| Proline | 0·76 | 0·37 | 0·14 | 0·29 |

Solvents: *A*, phenol/ethanol/water (3:1:1); *B*, *n*-butanol/pyridine/water (1:1:1); *C*, *n*-butanol/water/formic acid (10:6:1); *D*, *n*-butanol/water/acetic acid (6:3:1). (All proportions by volume.)

*Racemization of the natural amino-acid.* Natural pipecolinic acid (200 mg.), $Ba(OH)_2.8H_2O$ (400 mg.), and water (0·8 ml.) were sealed in a Pyrex tube and heated at 145° for 5 hr. The tube was washed out with water, the solution treated with $CO_2$, filtered from the precipitated $BaCO_3$, and concentrated to 5 ml. It was decolorized with a little charcoal, taken to dryness, dissolved in water (2 ml.), and the optical rotation determined: this was found to be zero. The solution was acidified with dilute $H_2SO_4$, filtered from the slight precipitate of $BaSO_4$, and passed through a column of Amberlite IR-100 resin. The effluent was free from amino-acid, which was then eluted with 0·5 N-$NH_3$. The eluate was concentrated (10 ml.), decolorized with charcoal and taken to dryness. The residue (185 mg.) was recrystallized from dry methanol and acetone, m.p. (microstage) 236° (decomp.).

The product of the racemization was chromatographically indistinguishable from the natural amino-acid and from the synthetic pipecolinic acid. It appeared identical in appearance and properties with the synthetic substance, and the melting point was not depressed by admixture. X-ray powder photographs (prepared by Miss A. A. Milne) showed the same pattern for the synthetic and racemized natural substances, and a different pattern for the original natural preparation (Fig. 2).

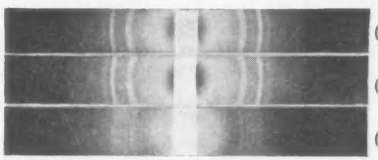


Fig. 2. X-ray powder photographs (paper negative) of pipecolinic acid specimens (Fe-filtered Co radiation; camera diameter 9 cm.). (*a*) Synthetic preparation; (*b*) natural preparation racemized; (*c*) natural preparation as obtained from clover ( × 1).

*Hydantoin of pipecolinic acid.* Synthetic pipecolinic acid (129 mg.) was converted to the hydantoin by treatment with KCNO (Leithe, 1932). The product was extracted from the reaction mixture with benzene, which was then removed and the residue (57 mg.), dissolved in $CHCl_3$, was purified by passage through a column of activated alumina followed by recrystallization from a mixture of benzene and light petroleum (b.p. 60–80°), m.p. (microstage) 120°. (Found: C, 55·3; H, 6·6; N, 18·0. Calc. for $C_5H_{10}O_2N_2$: C, 54·7; H, 6·5; N, 18·2%.) A product similarly obtained from the racemized natural preparation also had m.p. 120°, undepressed on admixture.

*Occurrence of pipecolinic acid in other plants*

Specimens of a number of plants were collected and extracted fresh in 50 g. quantities with 80% (v/v) aqueous ethanol (500 ml.) for about 10 min. in a top-drive macerator; the extracts were filtered from the insoluble material which was washed with solvent. The combined extract and washings were then treated by the method described above in the identification of amino-acids from clover leaves. Two-dimensional chromatograms were prepared by the same technique and using the same solvents.

The following plants were found to be relatively rich in pipecolinic acid: *Trifolium pratense*, *Vicia* sp. (cultivated broad bean), *Lathyrus* sp. (cultivated pea), *Laburnum anagyroides*, *Rosa canina* agg., *Daucus* sp. (cultivated carrot) *Allium ursinum*, *Luzula sylvatica*. The following contained a small amount: *Anemone nemorosa*, *Brassica* sp. (cultivated cabbage), *Geranium molle*, *Oxalis acetosella*, *Lupinus* sp., *Rubus fruticosus* agg., *Conium maculatum*, *Galium aparine*, *Petasites albus*, *Nicotiana affinis*, *Galeopsis speciosa*, *Rumex crispus*, *Urtica dioica*, *Fagus sylvatica*, *Allium* sp. (cultivated leek), *Juncus conglomeratus*, *Agrostis stolonifera*. Pipecolinic acid was not detected in the following plants: *Ranunculus repens*, *Chamaenerion angustifolium*, *Hedera helix*, *Sambucus nigra*, *Vaccinium myrtillus*, *Rhododendrum* sp., *Solanum tuberosum* (potato), *Digitalis purpurea*, *Taxus baccata*, *Dryopteris filix-mas*, *Sphagnum papillosum*. It was not detected in the following fungi: *Clitocybe nebularis*, *C. decastes*, *Fomes fomentarius* and *F. nigricans*.

Plants in the British flora are named according to the British Ecological Society Check List of British Vascular Plants (Clapham, 1946).

## DISCUSSION

No previous record has been found of the isolation of pipecolinic acid from natural sources. The amino-acid was first prepared in racemic form by Ladenburg (1891), and was resolved into optically active components by Mende (1896). Willstätter (1901) obtained it in a partially racemized form by the oxidation of conhydrine. Leithe (1932) prepared an optically active pipecolinic acid with a specific rotation considerably higher than that of Mende's preparation, and inferred that (+)-pipecolinic acid has D configuration since addition of acid altered the specific rotation in a negative direction (Lutz & Jirgensons, 1930, 1931, 1932). The $[\alpha]_D$ for the present natural preparation is less than half Leithe's value, and this suggests that partial racemization may have taken place at some stage in the isolation process.

The limited survey of the distribution of pipecolinic acid in plants shows that it is of fairly wide occurrence in angiosperms, although less abundant than $\gamma$-aminobutyric acid which it resembles in that

it appears not to be a constituent of proteins. No suggestion can be made at present as to its metabolic origin and fate; it appears, however, to have a closer structural affinity with lysine than with any other protein-forming amino-acid. A fairly diverse selection of angiosperms was examined, and pipecolinic acid was detected apparently sporadically throughout the group. In view of the structural relationship of the amino-acid with coniine and related alkaloids, it was thought that *Conium maculatum* might be a rich source, but only a small amount was detected. The richest source, relative to other amino-acids, was found to be *Luzula sylvatica*. It is of course probable that the amount of pipecolinic acid present in plant tissues will vary with the stage of development, and the fact that it was not detected in a particular plant does not exclude the possibility of its being present at some other stage: Biserte & Scriban (1950), investigating the development of free amino-acids in germinating barley, observed the appearance on the fourth day of an unknown ninhydrin-reacting substance which from the description appears to have been pipecolinic acid. The fact that the amino-acid has so far been detected only in angiosperms may not be significant, since only a few plants in other groups have been examined: Fowden (1951) has reported an unknown spot on paper chromatograms from algae which may possibly be attributed to pipecolinic acid.

## SUMMARY

1. An unknown amino-acid appearing on paper chromatograms of aqueous ethanol extracts of leaves of white clover (*Trifolium repens*) has been isolated by chromatographic methods using columns of cation-exchange resin and powdered cellulose.

2. The amino-acid has been shown, by analysis, racemization and comparison with a synthetic preparation to be pipecolinic acid (piperidine-2-carboxylic acid).

3. The natural amino-acid was optically active and had a laevo rotation, but was presumed to be partially racemized.

4. The behaviour of pipecolinic acid on paper chromatograms is described, and compared with that of nipecotinic acid.

5. A limited survey has been made of the occurrence of pipecolinic acid in plants.

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# Progressive Depletion in the Reduced Glutathione Content of the Blood following Acetoacetate Injections

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Glutathione has long been known to be an essential constituent of cells and to be indispensable for their normal activity since it serves to protect the cell enzymes. Several diabetogenic substances have been found to affect the reduced glutathione (GSH) content of various tissues. The injection of anterior pituitary preparations has been found to lower GSH content of tissues (Goss & Gregory, 1935; Gregory & Goss, 1939) and it has recently been shown by Conn, Louis & Wheeler (1948) that there is a direct correlation between the production of a transitory diabetes in man produced by injecting a purified adrenocorticotropic hormone and the blood GSH level. It has also been reported that the GSH of blood, tissues and, in particular, pancreas is decreased in diabetes (Campanacci, 1930). Leech & Bailey (1945) have investigated the effect of alloxan, and they found that the blood GSH may fall to near zero values on alloxan injection.

On the basis of these findings Lazarow (1949) attempted to correlate the diabetogenic action of a chemical compound with its ability to lower the GSH content of blood. He states that diabetes is usually developed in later stages of life, and hence it may not be unreasonable to suppose that gradual accumulation of some metabolic factors may be held responsible for a progressive degeneration of the $\beta$ cells of the pancreas. Attempts have been made in this laboratory to show that the intermediary fat metabolites, namely 'ketone bodies', might play the role of such a factor. Acetoacetate and $\beta$-hydroxybutyrate have already been shown to produce hyperglycaemia and to lower the potency of pancreatic insulin (Nath & Brahmachari, 1944, 1949) and to cause lowering of glucose tolerance and a rise of blood lactic acid (Nath & Chakrabarti, 1950, 1951).

In some preliminary observations it has already been shown from this laboratory (Nath & Hatwalne, 1950) that acetoacetate has some effect in lowering the GSH content of blood in rabbits. It is interesting to note that after the publication of our letter, Illing, Gray & Lawrence (1951) found that there was a significant reduction in glutathione in the diabetics with severe ketosis.

This paper gives an account of experiments on rabbits under various conditions.

## EXPERIMENTAL

Thirty male rabbits weighing from 1·5 to 2 kg. were chosen, and the normal GSH content of their blood was determined by the method of Woodward & Fry (1932). For this purpose 1·5 ml. of blood were withdrawn from the marginal ear vein of the rabbit by means of a syringe and immediately mixed with water and only reduced glutathione determined. The animals were fed not more than 2 hr. prior to taking a sample of blood for analysis to ensure that they were not starved. After the initial determination of GSH the rabbits were injected subcutaneously with the different doses of acetoacetate and GSH was estimated periodically.

Sodium acetoacetate was prepared in the usual way in the laboratory, other chemicals required being of the B.D.H. Analar quality.

Blood sugar was determined by the method of Hagedorn & Jensen (1923).

## RESULTS

First, the effect of a single injection of acetoacetate in doses of 300 mg./kg., 200 mg./kg. and 100 mg./kg. was studied on three groups of six rabbits each for a period of 3 hr., and a GSH determination was also made at the end of 24 hr. Blood sugar was determined simultaneously in the case of the first group alone (300 mg./kg.). It will be noted from Fig. 1 that the maximum drop in GSH is observed at the end of 1·5 hr after the injection and that it returns to almost normal values after 3 hr. The blood-sugar curve shows that the fall in GSH is accompanied by a rise in blood sugar.

If the injection of the same doses of acetoacetate were repeated on consecutive days results as depicted in Fig. 2 were obtained. Every day GSH was determined 1·5 hr. after the injection of acetoacetate since it was known from the previous experiment that the maximum effect could be observed only after that time. It can be seen from Fig. 2 that the GSH level falls to zero on the third, fourth and fifth days, respectively, with successively decreasing doses. Injections were discontinued from the sixth day and it was found that it took several days more for the GSH to return to normal.

The effect of smaller but gradually increasing doses of acetoacetate was also studied. For this purpose twelve rabbits were selected and injected with 50 mg./kg. of sodium acetoacetate for the

initial 10 days; 100 mg./kg. for the next 10 days and then 150 mg./kg. for a further 10 days. The GSH was determined after ten, twenty and thirty injections, i.e. on the eleventh, twenty-first and thirty-first day, before the next injection of sodium acetoacetate, and after making sure that the rabbits were fed within 2 hr. of the withdrawal of the blood.

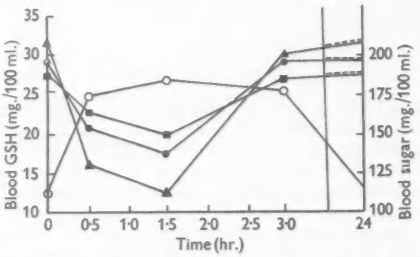


Fig. 1. Effect of single massive subcutaneous dose of acetoacetate on blood GSH content. ■—■, 100 mg./kg.; ●—●, 200 mg./kg.; ▲—▲, 300 mg./kg.; ○—○, blood-sugar curve.

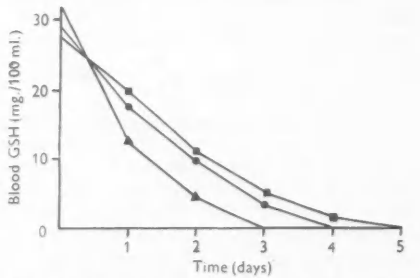


Fig. 2. Effect of repeated daily injection of acetoacetate on blood GSH content. ■—■, 100 mg./kg.; ●—●, 200 mg./kg.; ▲—▲, 300 mg./kg.

Results are represented in Fig. 3. There is a steep fall in GSH at the end of the first 10 days, and subsequently a further gradual fall can be noted. At the end of the experiment the values have fallen to slightly less than 30 % of the original.

To ascertain whether bleeding has any effect on the GSH content, 1·5 ml. of blood were withdrawn twice daily for a period of 5 days from six rabbits and GSH determined. It was found that there was no effect on the GSH content for 5 days.

## DISCUSSION

The experimental data presented indicate that acetoacetate causes a fall in the blood GSH content, although it is not as sharp and rapid as is reported in the case of alloxan (Leech & Bailey, 1945). It is only natural that this should be so since acetoacetate is an intermediary fat metabolite which normally occurs in very minute proportions in the system. Repeated daily injection brings the blood GSH value to zero in 3–5 days, less time being required with higher dose. Though alloxan causes an immediate fall of the GSH content to zero, nearly 1·5 hr. are required to obtain the maximum effect of an acetoacetate injection. The mode of injection may also account for this difference since alloxan is given intravenously and acetoacetate subcutaneously.

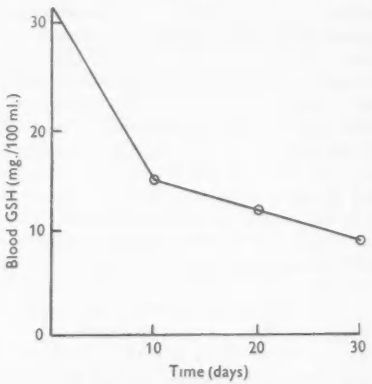


Fig. 3. Effect of smaller but gradually increasing doses of acetoacetate for a number of days on blood GSH.

The fall in the blood GSH caused by the injection of a 300 mg./kg. dose of acetoacetate is accompanied by a simultaneous transitory hyperglycaemia. It appears that blood sugar may have some relation with GSH and the presence of a substance like acetoacetate, which becomes toxic at higher concentrations. This also supports the hypothesis put forward by Nath & Brahmachari (1944) that accumulation of acetoacetate may be a predisposing cause of diabetes.

The decrease in GSH content in the blood, as a result of acetoacetate accumulation, could be due to many factors. It has been suggested that sodium depletion may be a contributory factor (Grunert & Phillips, 1949; Illing *et al.* 1951). Since we have used a sodium salt of acetoacetic acid, sodium depletion is unlikely to be the contributory factor in our experiments. It is probable that the fall is due to increased requirement as also suggested by Illing *et al.* (1951).

## SUMMARY

1. The effect of acetoacetate injections (single and repeated) on the reduced glutathione (GSH) content of the blood of rabbits was studied. Aceto-

acetate was consistently found to lower the GSH content, the fall in GSH being greater as the dose of acetoacetate increased.

2. The GSH level could be reduced to zero after repeated injection of acetoacetate.

The authors record their grateful thanks to the Indian Council of Medical Research for the award of a Research Fellowship to one of them (V.G.H.).

They are also thankful to Shri V. K. Sahu for his help in determining the blood-sugar values recorded in Fig. 1.

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# Studies on Alloxan Diabetes

## 1. INCREASE OF SUSCEPTIBILITY CAUSED BY ACETOACETATE

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Acetoacetate, which has been found in this laboratory to cause hyperglycaemia (Nath & Brahmachari, 1944), to reduce glucose tolerance (Nath & Chakrabarti, 1950), to raise blood lactic acid (Nath & Chakrabarti, 1951) and to inactivate insulin both *in vitro* and *in vivo* (Nath & Brahmachari, 1946), has recently been reported to cause decrease in the reduced glutathione (GSH) content of blood by Nath & Hatwalne (1950) and Nath, Hatwalne & Gadgil (1953). Griffiths (1948, 1950), in his studies on the production of diabetes by the administration of uric acid, noticed that diabetes could be produced in the animals only when the blood GSH level could be lowered by a diet deficient in cysteine and methionine and by feeding a large amount of ascorbic acid, which was shown by Prunty & Vass (1943) to lower the glutathione content of the blood, and to increase the susceptibility of rats to alloxan diabetes (cf. Lazarow, 1946). The increased sensitivity of starved animals to the diabetogenic action of alloxan, as noticed by Kass & Waisbren (1945), might be due to the fall of blood GSH which takes place during starvation (Hirano, 1934). It is also worth noting that animals depleted in

Table 1. *Effect of acetoacetate injection on the lowering of blood GSH and increase of blood sugar*

(Average values with the calculated mean deviations.)

| Group no. | No. of animals in the group | Blood GSH (mg./100 ml.) Initial | Blood GSH (mg./100 ml.) After injection of acetoacetate for 5 weeks (150 mg./kg.) | Blood sugar of the treated animals (mg./100 ml.) |
|---|---|---|---|---|
| I | 6 | 28·8±3·6 | 2·7±0·1 | 131·8±4·9 |
| II | 6 | 27·8±3·8 | 2·5±0·2 | 131·0±3·1 |
| III | 6 | 31·3±3·9 | 2·7±0·2 | 134·0±2·7 |
| IV | 6 | 30·7±2·9 | 2·5±0·7 | 133·0±3·6 |
| V | 6 | 28·8±3·4 | 2·7±0·5 | 133·0±4·4 |

blood GSH by a sodium-deficient diet have also been shown to have increased susceptibility to alloxan (Grunert & Phillips, 1949).

Archibald (1945) and Lazarow, Patterson & Levy (1948) have shown that when alloxan is administered it undergoes destruction partly by the glutathione of the blood and partly by other mechanisms. When the dose is high, some of it reaches the $\beta$ cells of the pancreas and reacts with the glutathione of these $\beta$ cells and even causes cell death by reacting with the sulphydryl groups of the protein and the enzymes. It is, therefore, expected that any substance which would decrease GSH in the body would also increase the susceptibility to alloxan.

This work was undertaken to see whether acetoacetate, which can produce various diabetic symptoms and can cause reduction of blood GSH, can cause an increase in the susceptibility to alloxan.

## EXPERIMENTAL

Thirty male rabbits weighing 1·5–2 kg. were selected and divided into five groups of six and their normal blood GSH was determined by the method of Woodward & Fry (1932). They were then injected subcutaneously with 150 mg./kg. of sodium acetoacetate daily for 5 weeks, when usually all the animals had turned over from their initial hypoglycaemic to the hyperglycaemic state (Nath & Chakrabarti, 1950). Blood sugar was estimated by the method of Hagedorn & Jensen (1923). After 5 weeks, when the animals in all the five groups were found to have very low blood GSH, those in one group were kept as control while those in other groups were injected intravenously with different doses of alloxan after allowing them to fast for about 18 hr. Twenty-four normal rabbits were also used to ascertain the diabetogenic and lethal doses of alloxan on the normal animals in our laboratory.

## RESULTS AND DISCUSSION

Table 1 shows that animals in all the groups show a slight rise in the fasting blood-sugar level and a sharp decline in the blood GSH content on injection of acetoacetate for 5 weeks. Urine of all the animals was analysed at this time and none was found to contain glucose, ketone bodies or albumin.

It will be evident from Table 2 that although a dose of 25 mg./kg. of alloxan is absolutely without any effect, those having 50 mg./kg. showed a definite tendency to have hyperglycaemia without glycosuria. A dose of 75 mg./kg., however, produced marked increase in the blood sugar with glycosuria and acetonuria and also albuminuria in some cases. A dose of 100 mg./kg. was found fatal to about 80 % of the animals who died within 48 hr., the only one surviving showing blood sugar as 410 mg./100 ml. and 4·5 % sugar in urine. The test for acetone in the urine was strongly positive and the albumin content high.

Table 2. *Effect of different doses of alloxan on normal animals and on animals whose blood GSH was depleted by acetoacetate injections*

(Average values with the calculated mean deviations.)

| Group no. | Nature of animals | No. of animals in the group | Dose of alloxan (mg./kg.) | Blood sugar 48 hr. after alloxan injection (mg./100 ml.) | Pathological constituents in urine | | | Remarks |
|---|---|---|---|---|---|---|---|---|
| | | | | | Sugar (%) | Acetone bodies | Albumin | |
| I | GSH depleted | 6 | 0 | 131·7±3·5 | 0 | – | – | |
| II | GSH depleted | 6 | 25 | 131·0±3·1 | 0 | – | – | No effect of alloxan |
| III | GSH depleted | 6 | 50 | 152·0±6·8 | 0 | – | – | Hyperglycaemia without glycosuria |
| IV | GSH depleted | 6 | 75 | 212·0±13·6 | 2·0±0·8 | + | + | One animal died within 48 hr. The remaining animals were diabetic. Glycosuria and hyperglycaemia continued for 5 weeks in all the animals |
| V | GSH depleted | 6 | 100 | 410·00 | 4·5 | + + | + + | High glycosuria and hyperglycaemia in the only animal surviving for 5 days. All other animals died within 48 hr. |
| VI | Control | 4 | 125 | 163·5±2·5 | 0 | – | – | Hyperglycaemia without glycosuria |
| VII | Control | 4 | 130 | 186·25±12·8 | 0·775±1·1 | + | + | Only two animals became diabetic |
| VIII | Control | 4 | 135 | 206·75±23·5 | 2·13±0·25 | + | + | Only three animals became diabetic |
| IX | Control | 4 | 140 | 259·00±5·3 | 2·7±0·13 | + | + | All the animals became diabetic |
| X | Control | 4 | 200 | 335·00±2·6 | 3·4±0·3 | + | + + | Severe diabetes |
| XI | Control | 4 | 225 | . | . | . | . | All animals died within 48 hr. |

From Table 2 it will also be seen that the diabetogenic dose of alloxan in normal animals, as found in this laboratory, ranges between 130 and 140 mg./kg. and the lethal dose is observed to be in the neighbourhood of 225 mg./kg. It is interesting to note that the diabetogenic and lethal doses of alloxan in acetoacetate-treated animals, having blood GSH in the neighbourhood of 3 mg./100 ml., are only 75 and 100 mg./kg., respectively. Continued injection of acetoacetate for a few weeks has thus a definite effect in making the animals susceptible to alloxan.

It may be mentioned here that the diabetogenic and lethal doses of alloxan in normal animals, as quoted by Lukens (1948) and others, are 150–200 and 300 mg./kg., respectively, which are somewhat higher than the values observed by us. The disparity may be explained by the difference in diet, climate and breed of the animals.

## SUMMARY

1. Rabbits injected subcutaneously with 150 mg./kg. sodium acetoacetate daily for 5 weeks show a slight rise in blood sugar, and an enormous fall in reduced glutathione in blood.

2. The susceptibility of these treated animals to alloxan is increased to a great extent, 75 mg./kg. being the diabetogenic dose and 100 mg./kg. the lethal dose, as compared with 130–150 and 225 mg./kg., respectively, for controls.

The authors record their grateful thanks to the Indian Council of Medical Research for the award of a Research Fellowship to one of them (V.G.H.).

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# The Assimilation of Amino-acids by Bacteria

## 14. NUCLEIC ACID AND PROTEIN SYNTHESIS IN *STAPHYLOCOCCUS AUREUS*

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(*Received* 22 *May* 1952)

If washed suspensions of *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) are incubated in buffered salt solution containing glucose and glutamic acid, free glutamic acid accumulates to a high concentration within the cell (Gale, 1947*a*, *b*). If certain other amino-acids, such as cysteine or alanine, are added singly to the incubation mixture, the accumulation of free glutamic acid within the cells is reduced while peptides of glutamic acid appear in the external medium (Gale & Van Halteren, 1951). If a complete mixture of amino-acids and glucose is present, then the cells no longer accumulate free glutamic acid but the combined glutamate of the cellular substance increases, glutamic acid from inside and outside the cell becoming directly incorporated into this combined glutamate fraction (Gale, 1951*b*).

In the investigations so far described in this series, no account has been taken of the nucleic acid content of the cells or of its variation. In the present work, the nucleic acid content has been studied and the effect of the addition of purines and

pyrimidines to the incubation mixture tested on the various stages of glutamic acid assimilation. Malmgren & Heden (1947) investigated the variation of 'nucleotide' content of bacterial cells during the period of growth; they used the absorption by the cells of light of wavelength 257 mμ. as a measure of 'nucleotide' content and found that the synthesis of this 'nucleotide' was greatest during the first generation time, the content falling again during the later stages of growth. Comparison of the curves obtained for 'nucleotide' synthesis and rate of growth for a wide variety of bacteria suggested that the 'nucleotide' content of the cell played a controlling role in its rate of protein synthesis. Caldwell, Mackor & Hinshelwood (1950) found that the deoxyribonucleic acid content of *Bacterium lactis aerogenes* (presumably *Aerobacter aerogenes*) was an approximately constant component of the cell, whereas the ribonucleic acid content varied over a wide range, and that the rate of growth of the cell was proportional to the ribonucleic acid content. Caldwell & Hinshelwood (1950) put forward theoretical considerations suggesting that nucleic acid guides the order in which amino-acids are laid down during protein synthesis by bacteria; they further suggest that protein, in turn, guides the order in which nucleotide units are combined in nucleic acid synthesis. Evidence and theories concerning the role of nucleic acid in protein synthesis have recently been reviewed by Chantrenne (1952).

## METHODS

*Organism.* The organism used throughout these studies was *Staph. aureus* Duncan, the strain used for earlier studies in this series and originally isolated from pus.

*Growth medium and preparation of suspensions.* The organism was grown overnight at 30° in the 'deficient medium *B*' previously described (Gale, 1951*a*) which consists of a salt medium with glucose, Marmite and arginine added. The organisms were harvested on the centrifuge, washed once with distilled water, and made up in distilled water to a suspension density of approximately 20 mg. dry wt. of cells/ml. Dry weights were determined turbidimetrically on a Hilger absorptiometer previously calibrated against the organism used. The cell suspension was diluted 1:10 during incubation with amino-acid mixtures, etc., washed and concentrated on the centrifuge to the original strength for estimation of glutamic acid, etc.

*Growth tests.* The nutritional requirements of *Staph. aureus* Duncan were determined in synthetic media according to the general method of Gladstone (1937). A complete medium was prepared containing twenty amino-acids (as below), thiamine, nicotinamide and glucose; further tubes of media were prepared to test the effect of the omission of each amino-acid one at a time; all tubes were inoculated with a standard inoculum of $3–10 \times 10^3$ cells/10 ml. medium and growth inspected at 24 hr. intervals during incubation at 37°.

*Amino-acid solutions.* For experiments designed to test the effect of various combinations of amino-acids, 0·04 M solutions of glycine and of the L isomers of the following were prepared: aspartic acid, asparagine, glutamic acid, tyrosine, tryptophan, phenylalanine, histidine, lysine, arginine, methionine, proline, hydroxyproline, serine, cysteine, cystine, alanine, threonine, leucine, isoleucine and valine. Each solution was adjusted to pH 7·0 and diluted 1:30 in the incubation mixture unless otherwise stated. When a complete mixture was required, a solution (*A*) was prepared containing all the above amino-acids, each at a concentration of 2·0 mg./ml., the pH adjusted to 7·0 and, except where otherwise stated, diluted 1:10 in the incubation mixture.

*Purine and pyrimidine mixture.* Solutions of each of the following purines or pyrimidines at a concentration of 1·0 mg./ml. were prepared: adenine, xanthine, hypoxanthine, guanine, thymine, cytosine and uracil. A mixture (solution *P*) was also prepared containing all these substances, each at a concentration of 1·0 mg./ml.; only a small amount of cytosine was available and this was omitted from some experiments where it was found to have no significant effect, in such cases the purine-pyrimidine solution being noted as mixture *P'*. All these solutions were diluted 1:50 in the incubation mixture.

*Amino-acid estimations.* Free glutamic acid, arginine and lysine were estimated manometrically by use of the corresponding specific decarboxylase preparations (Gale, 1945*a*, *b*). Total amino-acid contents of cells and precipitates were determined by the same method applied to hydrolysates obtained after 18 hr. hydrolysis in boiling 6 N-HCl, excess acid being removed *in vacuo*. In preparations such as cell suspensions which contained both free and combined glutamic acid, the latter was calculated from the difference between the free and total contents.

*Accumulation of free glutamic acid within cells, increase in combined glutamate fraction, rates of fermentation and respiration.* These have been studied by the methods described in previous papers of this series (Gale, 1947*a*; 1951*a*, *b*).

*Precipitation and estimation of protein.* Suspensions of cells, after washing, were precipitated in the cold with trichloroacetic acid (TCA) to a final concentration of 5% (w/v). After standing for not less than 2 hr. in the cold, the precipitates were centrifuged down, washed once with cold 5% TCA, suspended in water and hydrolysed for 18 hr. in boiling 6 N-HCl. The excess acid was removed *in vacuo* and the hydrolysates made up to a known volume after the pH had been adjusted to approx. 5. The combined glutamate content was taken as a measure of protein present (see below).

*Nucleic acid content of cells.* Mitchell (1950) has described a method for the estimation of 'nucleic acid' in suspensions of bacteria. This involves estimation of the absorption at 260 mμ. and calculation of the correction for light scattering from the absorption at 350 mμ. The method estimates all substances having an absorption at 260 mμ. and thus includes free purines, nucleotides, etc. Salton (1951) has shown that organisms may contain free purines and pyrimidines, and that these are released by treatment of the cells with cetyltrimethylammonium bromide (CTAB). Since we wished to estimate the nucleic acid content of the cells free from purines or nucleotides, we tested the application of Mitchell's method to cell suspensions which had been treated with CTAB under the conditions described by Salton (1951), followed by washing and resuspension in water. It became evident during the course of the work that the calculated scattering correction introduced inaccuracies,

especially when inhibitors were used which had a differential action on protein and nucleic acid synthesis. Consequently, a more direct method of estimation was sought.

The cell suspensions were precipitated in the cold with 5% TCA as for the estimation of protein. After washing with cold 5% TCA, a portion of the precipitate, equivalent to approx. 20 mg. dry weight of original cell suspension, was extracted three successive times with 2·0 ml. 5% TCA at 90° for 10 min. 90–95% of the nucleic acid was extracted in the first two treatments and no further extraction took place after the third treatment. The extracts were collected, combined and made up to 10·0 ml. with water. 1·0 ml. of the extract was then diluted to 10·0 ml. with water and the absorption at 260 mμ. determined on a Beckman spectrophotometer against a blank containing an equivalent amount of TCA. Caspersson (1936) and Malmgren & Heden (1947) used an extinction coefficient of 22 for 0·1% nucleic acid, but Magasanik & Chargaff (1951) found that hydrolysis of the nucleic acid to nucleotides resulted in an increase in absorption of 20–30%. The extraction procedure used in this work results in hydrolysis of the nucleic acid, and the extinction coefficient used by Caspersson is clearly too low. A value of 28 for 0·1% nucleic acid has been adopted as the result of calculations based upon the base analysis of a number of preparations of nucleic acid from *Staph. aureus* grown and treated in various ways described in this paper and the next of the series (Gale & Folkes, 1953).

*Quantitative estimation of purines and pyrimidines.* An amount of TCA extract containing approx. 3 mg. of nucleic acid was evaporated to dryness, taken up in 20 μl. 72% (w/v) $HClO_4$ and heated in a sealed tube at 100° for 2 hr. The hydrolysate was diluted with 20 μl. water, centrifuged to remove carbon, and 20 μl. taken for separation and estimation of the bases by the method of Smith & Markham (1950) as modified by Wyatt (1951).

## RESULTS

### *Conditions affecting protein synthesis*

*Amino-acid requirements of* Staph. aureus *Duncan.* Full growth of the organism takes place in 10–12 hr. at 37° in a synthetic medium containing nineteen naturally occurring amino-acids. Omission of the amino-acids one at a time from the medium shows that arginine, lysine, methionine and hydroxyproline have no effect on growth, whereas the remaining fifteen amino-acids are essential for growth to occur within 24 hr. of inoculation. No growth occurs up to 128 hr. in the absence of histidine, aspartic acid, valine, leucine, isoleucine, tyrosine, phenylalanine, tryptophan or threonine, whereas delayed growth occurs in the absence of alanine, glycine, proline, glutamic acid or serine. It is probable that growth occurring after 24 hr. and before 128 hr. is due to the selection of mutants which are able to synthesize the missing amino-acid in each case. The delay caused by omission of glutamic acid is approximately 40 hr. and it is possible that sufficient glutamic acid for growth may be produced by transamination from aspartic acid but the latter is completely essential even after 128 hr. incubation. The salt mixture used as a basal medium was free from ammonium salts; the addition of ammonium salts resulted in markedly slower growth in all cases.

*Effect of amino-acid mixtures on combined glutamate formation.* Table 1 summarizes the changes which occur in the free and combined glutamic acid contents of cells and supernatant when incubation takes place for 30 min. in buffered salt solution containing glucose and various combinations of amino-acids. It can be seen that, as the complexity of the incubation mixture increases, the amount of free glutamic acid accumulating within the cells progressively decreases, as does the amount withdrawn from the medium. The combined glutamate content of the cells decreases when incubation takes place in mixtures of a few amino-acids, and it is not until all the essential amino-acids are present in the medium that a significant increase in combined glutamate occurs. The further addition of the non-essential amino-acids makes little difference to the rate of increase of combined glutamate, but this may be due to a slightly inhibitory action of methionine since omission of this amino-acid from the complete mixture results in a significantly increased accumulation of combined glutamate. Table 1 also shows the effect of the omission from the complete amino-acid mixture of one or two of the amino-acids essential for growth. Omission of aspartic acid decreases but does not abolish the increase in combined glutamate obtained in 30 min., but progress curves carried out for a longer period (Fig. 1) show that such increases only occur during

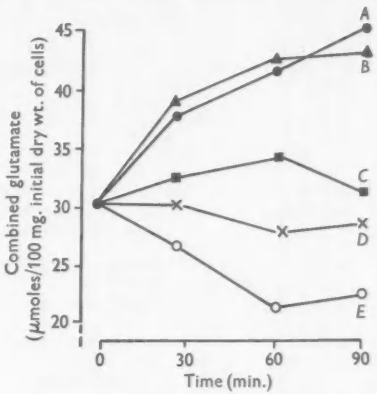


Fig. 1. Changes in the combined glutamate of cells incubated in buffered salt solution containing 1·0% glucose and additions. Additions: curve *A*, complete mixture of amino-acids; curve *B*, as (1) less lysine, arginine, methionine and proline; curve *C*, as (1) less aspartic acid; curve *D*, as (1) less cysteine; curve *E*, glutamic acid only. Conditions as for Table 2; incubation at 37°.

Table 1. *Effect of amino-acid mixtures on the accumulation of free and combined glutamic acid by washed suspensions of* Staphylococcus aureus

(Washed suspensions of *Staph. aureus* Duncan, at final suspension density of 2·0 mg. dry wt./ml., incubated for 30 min. at 37° in buffered salt solution containing 1% glucose and each amino-acid (marked +) to a final concentration of 1·34 $\mu$moles/ml. In cases marked (3) final amino-acid concentration is 4·02 $\mu$moles/ml. After incubation, cells harvested and washed on the centrifuge, and free and combined glutamic acid content determined for cells and supernatants. Experiments carried out with eight incubation mixtures on each occasion, one of these mixtures containing glucose and glutamic acid only; results expressed in terms of this control for comparative purposes.)

| Incubation mixture | | | | | | | | | | | | | | | | | | | | Relative increase in internal free glutamate of cells (100 = 34·5 $\mu$moles/30 min./100 mg. dry wt. of cells) | Change in combined glutamate of cells ($\mu$moles/30 min./100 mg. dry wt. of cells) | Relative change in free glutamate of supernatant (100 = 50·5 $\mu$moles/30 min./100 mg. dry wt. of cells) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Glucose | Glutamic acid | Aspartic acid | Cysteine | Alanine | Glycine | Histidine | Valine | Leucine | Tyrosine | Phenylalanine | Tryptophan | Threonine | Serine | Isoleucine | Arginine | Lysine | Proline | Methionine | | | | |
| + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | + 6·5 | − 1·6 | + 4 |
| + | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | +100 | − 5·8 | −100 |
| + | + | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | + 50 | − 5·2 | − 33 |
| + | + | 0 | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | + 77 | − 5·8 | −102 |
| + | + | + | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | + 37 | − 5·2 | − 34·5 |
| + | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | + | 0 | 0 | 0 | | +106 | − 6·5 | −109 |
| + | + | 0 | 0 | 0 | 0 | 0 | + | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | +110 | − 6·3 | −103 |
| + | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | + | + | + | + | | + 92 | − 5·6 | − 96 |
| + | + | + | + | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | + 34 | − 8·3 | − 35 |
| + | + | + | + | + | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | + 22·5 | − 4·3 | − 39 |
| + | + | + | + | + | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | + | 0 | 0 | 0 | 0 | 0 | | + 25 | − 3·1 | − 62·5 |
| + | + | 0 | + | + | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | + | 0 | 0 | 0 | 0 | 0 | | + 66 | − 5·8 | − 80 |
| + | + | + | 0 | + | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | + | 0 | 0 | 0 | 0 | 0 | | + 38 | −10·9 | − 70 |
| + | + | + | + | + | + | 0 | + | + | 0 | 0 | 0 | 0 | + | 0 | 0 | 0 | 0 | 0 | | + 35 | − 6·8 | − 54 |
| + | + | + | + | + | + | 0 | + | + | + | + | 0 | 0 | + | 0 | 0 | 0 | 0 | 0 | | + 34 | − 3·7 | − 47 |
| + | + | + | + | + | + | 0 | + | + | + | + | + | 0 | + | 0 | 0 | 0 | 0 | 0 | | + 35 | − 5·2 | − 35 |
| + | + | + | + | + | + | 0 | + | + | + | + | + | + | + | 0 | 0 | 0 | 0 | 0 | | + 21 | − 0·8 | − 43 |
| + | + | + | + | + | + | 0 | + | + | + | + | + | + | + | + | 0 | 0 | 0 | 0 | | + 22 | + 0·6 | − 35 |
| + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | 0 | 0 | 0 | 0 | | + 4·3 | + 2·7 | − 15 |
| + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | 0 | 0 | 0 | | + 3·2 | + 2·8 | − 14 |
| + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | | − 6·0 | + 3·4 | − 7 |
| + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | 0 | | − 2·5 | + 3·9 | . |
| + | + | + | + | + | + | + | + | + | + | + | 0 | 0 | + | + | + | + | + | + | | − 0·6 | + 3·5 | − 7 |
| + | + | + | + | + | + | + | 0 | 0 | + | + | + | + | + | 0 | + | + | + | + | | + 10 | + 2·7 | − 5 |
| + | + | 0 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | | + 34 | + 2·2 | − 47 |
| + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | 0 | + | + | + | | + 2 | + 2·1 | − 27 |
| + | + | + | + | + | + | + | + | + | 0 | 0 | + | + | + | + | + | + | + | + | | + 13 | + 1·4 | . |
| + | + | + | + | + | + | 0 | + | + | + | + | + | + | + | + | + | + | + | + | | + 18 | + 0·2 | − 8 |
| + | + | 0 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | | + 9 | − 0·3 | − 15 |
| + | + | 0 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | 0 | | + 15 | − 3·2 | − 7 |
| + | + | 3 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | | − 16 | + 3·7 | − 21·5 |
| + | + | + | 3 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | | − 14 | + 4·5 | − 12·6 |
| + | + | + | + | 3 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | | − 14 | + 4·5 | − 14 |

the early part of the incubation, the complete mixture of essential amino-acids being necessary for continued production of combined glutamate. On incubation for 90 min. or more, a mixture of all the natural amino-acids is slightly more effective than a mixture of the essential amino-acids only.

The amino-acids found by Gale & Van Halteren (1951) to decrease the accumulation of free glutamic acid within the cells included aspartic acid, cysteine and alanine. Table 1 shows that increasing the concentration of cysteine, aspartic acid or alanine in the incubation mixture results in an increase in the rate of accumulation of combined glutamate in the cells.

*Nature of the combined glutamate fraction.* Table 2 shows that 90 % of the combined glutamate of the cells, before and after incubation with glucose and a complete mixture of amino-acids, is precipitated by 5 % TCA in the cold. Analysis of the material obtained by disintegrating the cell suspension on a Mickle vibrator (Mickle, 1948) shows that at least 92 % of the TCA-precipitable combined glutamate resides in the 'soluble' portion of the cells that is not centrifuged down at 4000 *g* in 20 min.

It is possible that an increase in combined glutamate of protein-like materials could occur without a concomitant increase in whole protein. Consequently, the TCA precipitate was analysed for lysine, arginine, amino nitrogen and total nitrogen in addition to glutamic acid. Table 3 shows a series of such determinations for TCA precipitates obtained from cells treated in various ways described below and in the next paper (Gale & Folkes, 1953). The precipitates obtained by treatment of cells with cold 5 % TCA contain nucleic acid as well as protein and some of the treatments listed in Table 3 (incubation in the presence of chloramphenicol in particular (Gale & Folkes, 1953)) result in an increase in nucleic acid but not in protein content; consequently total nitrogen values will not show variations which can be correlated with protein content only. Table 3 shows that combined glutamate estimations give a good indication of variations in protein content, and that the total nitrogen values correlate least well, of the quantities measured, with the combined glutamate values. In general, increases in protein content have been

Table 2. *Nature of combined glutamate fraction of* Staphylococcus aureus

(Cells harvested from deficient medium; estimations carried out on (*a*) cells as harvested; (*b*) cells after incubation for 1 hr. at 37° in buffered salt solution containing glucose and amino-acid mixture *A*. Cells were washed after incubation, and free and combined glutamic acid estimations carried out on the cell suspensions and on precipitates obtained therefrom by treatment with 5 % TCA in the cold. 'Soluble' and insoluble fractions of cells obtained by centrifuging cell debris at 4000 *g* for 20 min. after disintegration of cells on the Mickle machine (Mickle, 1948).)

| Exp. no. | Preparation | Combined glutamate (μmoles/100 mg. dry wt. of cell) (*a*) | (*b*) | Increase (*b* − *a*) |
|---|---|---|---|---|
| 1 | Intact cells | 28·8 | 37·6 | 8·8 |
| | (i) TCA precipitate | 25·6 | 34·1 | 8·5 |
| | (ii) TCA-soluble fraction | 3·2 | 3·8 | 0·6 |
| 2 | Mickle disintegrate | | | |
| | (i) Insoluble fraction | 1·8 | 2·2 | 0·4 |
| | (ii) 'Soluble' fraction | 19·9 | 23·6 | 3·7 |
| | (iii) TCA precipitate of 'soluble' fraction | 19·8 | 23·4 | 3·6 |

Table 3. *Analyses of protein precipitates from* Staphylococcus aureus

(*Staph. aureus* Duncan grown in deficient medium, harvested and made into washed suspension. Cells incubated for 1 hr. at 37° in buffered salt medium with additions as below. After incubation, cells washed in water and precipitated with 5 % TCA for 2 hr. in the cold; precipitates washed with cold 5 % TCA and then hydrolysed for 18 hr. in boiling 6 N-HCl; excess acid removed *in vacuo* and amino-acid determinations, etc. carried out on neutralized hydrolysates. Results expressed as percentage initial suspension. Abbreviations: *G*, glucose; *A*, complete mixture of amino-acids; *A* − *g*, complete mixture of amino-acids less glutamic acid; *P*, mixture of purines and pyrimidines; *CAP*, 30 μg. chloramphenicol/ml.)

| | Initial suspension (mg. N/100 mg. dry wt. of cells) | Additions to incubation mixture: *G* | *G*, *A* | *G*, *A*, *P* | *G*, *A* − *g*, *P* | *G*, *A*, *P*, *CAP* |
|---|---|---|---|---|---|---|
| Glutamic acid | 0·414 | 93 | 121 | 134 | 100 | 96 |
| Arginine | 0·441 | 96 | 125 | 136 | 108 | 101 |
| Lysine | 0·910 | 103 | 118 | 133 | 108 | 104 |
| Amino-N | 5·95 | 99 | 123 | 131 | 106 | 106 |
| Total-N | 8·86 | 98 | 120 | 134 | 103 | 115 |
| Nucleic acid | — | 95 | 112 | 148 | 113 | 178 |

estimated by determination of the combined glutamate content of the TCA precipitates obtained from the cells.

*Effect of addition of purine and pyrimidine mixtures.* The synthesis of cellular protein described above takes place when the cells are incubated in the presence of glucose and a mixture of amino-acids. Fig. 2 shows the effect of adding the purine-pyrimidine mixture *P′* to the incubation medium; the rate of protein synthesis is significantly accelerated (see Table 3 also). In a series of twelve observations under the conditions holding for the

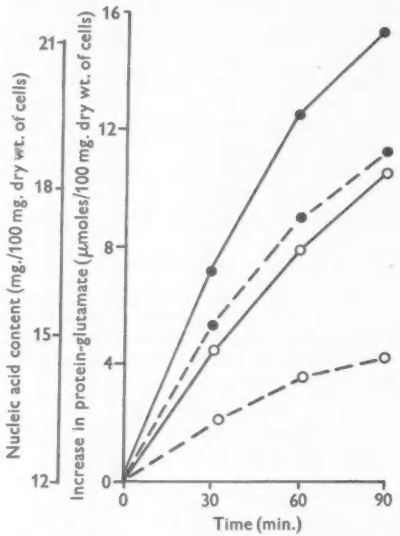


Fig. 2. Increase in nucleic acid (broken lines) and protein (full lines) of *Staph. aureus* when incubated in buffered salt solution containing 1% glucose and the complete mixture of amino-acids *A* with (●) and without (○) the purine-pyrimidine mixture *P′*.

experiment shown in Fig. 2, the mean acceleration of protein synthesis resulting from the addition of mixture *P′* has been 49·5% of the control without *P′* (range 18–112%).

*Effect of concentration of amino-acid mixture.* In the experiments described above, the concentration of the amino-acid mixture solution *A* was such as to give a final concentration of 0·2 mg. of each amino-acid/ml. medium. Fig. 3 shows the effect of increasing the concentration of the mixture *A* on the rate of protein synthesis with and without the addition of mixture *P* to the medium. It can be seen that the protein-synthesizing system is saturated at a much lower amino-acid concentration in the absence of *P* than in its presence, and that, at a high amino-acid concentration, the presence of *P* may increase the rate of protein synthesis by as much as 300%.

*Conditions affecting nucleic acid synthesis*

*Effect of amino-acids.* Fig. 4*a* shows that there is little or no increase in the nucleic acid content of cells if these are incubated in the presence of glucose alone, mixture *P* alone, or glucose and mixture *P*. However, if amino-acids are added to the incubation mixture, a significant increase in nucleic acid occurs. Glycine has a marked effect, but most amino-acids added alone have only a small effect; simple mixtures of four or five amino-acids have some effect but, in general, the larger the number of amino-acids present, the greater the stimulation of

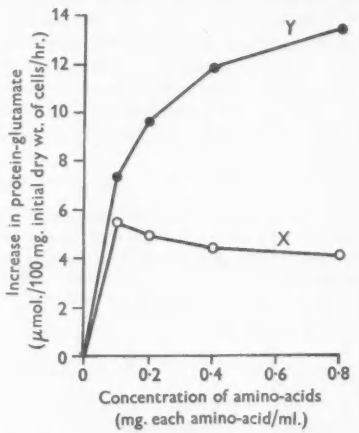


Fig. 3. Effect of amino-acid concentration on rate of protein synthesis by *Staph. aureus* Duncan in presence (●) and absence (○) of purine-pyrimidine mixture *P*. Cells grown for 18 hr. at 30° in deficient medium; made into washed suspension and incubated for 1 hr. at 37° in buffered salt solution containing 1·0% glucose and amino-acid mixture *A* at concentrations shown, with (curve *Y*) and without (curve *X*) purine-pyrimidine mixture *P* at final concentration of each component = 0·02 mg./ml. Protein assayed by combined glutamate content of TCA precipitate.

nucleic acid formation. A mixture of all the amino-acids essential to growth gives a marked stimulation, which is slightly increased by the further addition of the non-essential amino-acids. Increasing the concentration of the complete amino-acid mixture beyond that quoted in Fig. 4 does not further increase the formation of nucleic acid. Omission of one essential amino-acid, such as aspartic or glutamic acid, from the complete mixture results in a greatly decreased effect. Comparison of the effects shown in Table 1 and Fig. 4 suggests that the action of

amino-acids on nucleic acid formation can be correlated with their ability to promote protein synthesis.

*Effect of purines and pyrimidines.* Fig. 2 shows progress curves for the increase in the nucleic acid content of cells incubated with a complete amino-acid mixture with and without mixture *P'*. There is a small increase in nucleic acid in the absence of added *P'* and this is greatly increased when *P'* is added to the mixture. The growth of *Staph. aureus* Duncan is not nutritionally exacting towards purines or pyrimidines under aerobic conditions, and Salton (1951) has shown that Staphylococci contain free purines and pyrimidines within the cell, so that the small increase in nucleic acid that occurs on incubation with amino-acids and glucose may take place at the expense of purine derivatives already present within the cell or synthesized during the incubation.

Fig. 4*b* shows that the nucleic acid synthesis occurring on incubation with glucose and amino-acids is not greatly stimulated by the addition of any one purine or pyrimidine, whereas the complete mixture *P* has a marked effect. Omission of adenine, guanine, cytosine or thymine singly from the mixture *P* has little effect, whereas omission of uracil or xanthine results in a marked decrease in nucleic acid formation. The complete mixture without uracil has little more effect than that of the amino-acid mixture alone, and this can be correlated with the finding by Richardson (1936) that *Staph. aureus* strains are unable to synthesize uracil during growth under anaerobic conditions. Since cytosine was available in small quantities only, and its omission from mixture *P* had little or no effect on nucleic acid synthesis, it was not added in many of the later experiments (Gale & Folkes, 1953).

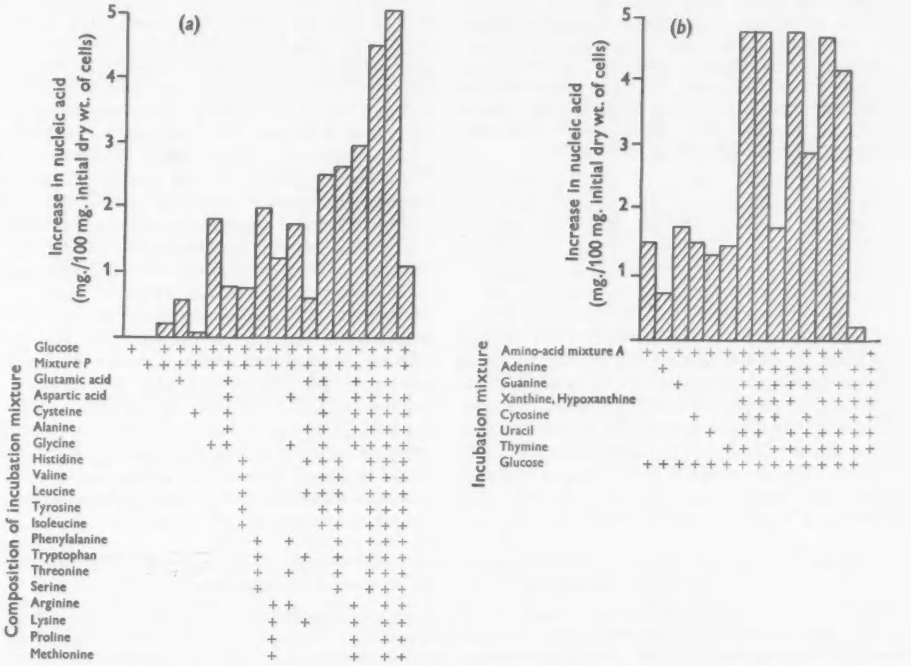


Fig. 4. Nucleic acid synthesis in *Staph. aureus* Duncan showing effect of addition of (*a*) amino-acids and (*b*) purines and pyrimidines to the incubation mixture. Cells grown for 16 hr. at 30° in 'deficient' medium; made into washed suspension and incubated, at final suspension density = 2·0 mg./dry wt./ml., for 1 hr. at 37° in buffered salt medium containing 1% glucose, amino-acids (final concentration of each component = 1·34 $\mu$moles/ml.), purines and pyrimidines (final concentration of each component = 0·02 mg./ml.); the presence of each component in the incubation mixture is indicated by +. After incubation, cells precipitated with 5% TCA in the cold, nucleic acid fraction extracted with hot 5% TCA and estimated spectrophotometrically.

No increase in nucleic acid has been observed in the absence of glucose in the incubation medium.

Table 4. *Composition of nucleic acid fraction of* Staphylococcus aureus

((*a*) *Staph. aureus* Duncan grown for 6 hr. at 37° in 'deficient' medium, harvested and made into washed suspension. (*b*) *Staph. aureus* Duncan grown for 16 hr. at 30° in 'deficient' medium, harvested and made into washed suspension. (*c*) Portion of suspension (*b*) after incubation for 1 hr. at 37° in buffered salt solution containing glucose, amino-acid mixture *A* and purine-pyrimidine mixture *P'*. In all cases, cells precipitated with 5% TCA in the cold, precipitates washed with cold 5% TCA, extracted three successive times with 5% TCA at 90° for 10 min., extracts collected and analyses performed on combined extracts. Results expressed as % dry wt. of initial cells.)

| | (*a*) | (*b*) | (*c*) |
|---|---|---|---|
| Total nucleic acid | 14·1 | 10·3 | 13·0 |
| Adenine | 1·34 | 0·86 | 1·07 |
| Guanine | 1·68 | 1·02 | 1·29 |
| Cytosine | 0·90 | 0·66 | 0·83 |
| Uracil | 0·625 | 0·535 | 0·675 |
| Thymine | 0·22 | 0·25 | 0·27 |

*Composition of the nucleic acid fraction.* Table 4 gives analyses of the purine and pyrimidine bases determined on hydrolysates of nucleic acid fractions obtained from *Staph. aureus* before and after incubation with the complete incubation mixture containing glucose, amino-acids and mixture *P'*, and also from cells harvested at two different 'ages' of culture. The major part of the changes in the nucleic acid would appear to reside in the ribonucleic acid fraction; this is in accordance with the findings of Caldwell *et al.* (1950) for *Bact. lactis aerogenes*.

*Relationships between nucleic acid content of Staphylococcal cells and their ability to synthesize cellular protein or to accumulate free glutamic acid*

Previous studies in this series have suggested that there is a metabolic connexion between the processes of free glutamic acid accumulation and of protein synthesis (Gale, 1951*b*; Gale & Van Halteren, 1951). A number of workers have suggested a connexion between protein synthesis and nucleic acid metabolism (Jeener & Brachet, 1944; Caspersson, 1947; Malmgren & Heden, 1947; Caldwell & Hinshelwood, 1950; Swensson, 1950; Jeener & Jeener, 1952; Chantrenne, 1952). In the course of the work described here and in the next paper (Gale & Folkes, 1953), it became clear that a number of methods had been obtained whereby the nucleic acid content of washed *Staph. aureus* cells could be altered; some of these are summarized in Table 5. Malmgren & Heden (1947) have described how the 'nucleotide' content of bacteria varies with the age of the culture so that cells with different nucleic acid contents can presumably also be obtained by harvesting the organisms at different phases of growth. It was therefore decided to use these various methods to test what relationship, if any, could be found between the nucleic acid content of the cells and their ability either to synthesize protein or to accumulate free glutamic acid.

Table 5. *Variation of rates of protein synthesis or accumulation of free glutamic acid with nucleic acid content of washed suspensions of* Staphylococcus aureus

(Suspensions of *Staph. aureus* Duncan incubated in buffered salt solution with additions as below (first treatment) for 45 min. at 37°. Cells centrifuged down, washed and estimations made of nucleic acid and protein-glutamate content. The remainder of each batch divided into two and incubated in buffered salt solution containing 1% glucose and (*a*) complete amino-acid mixture *A* for 1 hr. at 37°, or (*b*) 2·7 μmoles sodium glutamate/ml. for 15 min. at 37°. Increases in protein-glutamate and internal free glutamic acid estimated. Results expressed on basis of (i) 100 mg. dry wt. of cells as initially sampled, and (ii) 100 mg. dry wt. of cells allowing for the increase in protein content during the first treatment.)

| | First treatment | | | Second treatment | | | |
|---|---|---|---|---|---|---|---|
| | | Nucleic acid content at end (mg./100 mg. cells) | | (*a*) Increase in protein glutamate (μmoles/100 mg. cells) | | (*b*) Increase in free glutamic acid content (μmoles/100 mg. cells) | |
| Additions to salt solution | Protein-glutamate at end of treatment (μmoles/100 mg. initial dry wt. of cells) | (i) | (ii) | (i) | (ii) | (i) | (ii) |
| (1) Glucose, complete amino-acid mixture *A*, purine mixture *P'* + 30 μg. chloramphenicol/ml. | 28·8 | 17·1 | 17·4 | 6·91 | 7·08 | 12·1 | 12·4 |
| (2) As (1) with glutamic acid omitted from mixture *A* | 27·0 | 16·45 | 17·1 | 6·0 | 6·52 | 12·7 | 13·8 |
| (3) As (1) without chloramphenicol | 35·1 | 15·05 | 12·75 | 5·6 | 4·7 | (19·7 | 16·4) |
| (4) As (3) + 3000 units penicillin G/ml. | 32·4 | 13·75 | 11·75 | 6·2 | 5·68 | 15·2 | 13·8 |
| (5) Glucose, amino-acid mixture *A* | 32·4 | 12·7 | 11·5 | 6·9 | 6·3 | 16·5 | 14·1 |
| (6) Glucose only | 29·0 | 10·7 | 10·8 | 4·8 | 4·85 | 20·0 | 20·3 |
| (7) Initial suspension (first treatment omitted) | 29·4 | 10·5 | — | 4·04 | — | 21·2 | — |

*Alteration of nucleic acid content of washed cells.* A large batch of *Staph. aureus* was grown and harvested in the usual manner. The nucleic acid content and rates of fermentation, free glutamic acid accumulation and protein synthesis (in the absence of added purines or pyrimidines) were measured. Various portions of the suspension were then incubated for 45 min. under the conditions set out in Table 5, with the consequent changes in nucleic acid content also shown. Each portion of the suspension was then washed, divided into two, and the rates of protein synthesis or free glutamic acid accumulation determined under the appropriate conditions. The

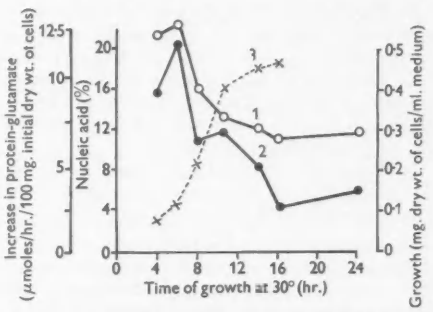
Increase in protein-glutamate (μmoles/hr./100 mg. initial dry wt. of cells)
Nucleic acid (%)
Growth (mg. dry wt. of cells/ml. medium)
Time of growth at 30° (hr.)
1
2
3

Fig. 5. Variation with age of culture in *Staph. aureus* Duncan of nucleic acid content (○), rate of protein synthesis (●) and growth (×). Growth took place in deficient medium at 30°. Samples of culture taken at intervals as shown, washed suspensions prepared and divided into two portions. Nucleic acid content determined on one; rate of increase in protein glutamate determined on other by incubation for 1 hr. at 37° in buffered salt solution containing 1% glucose and amino-acid mixture *A* (final concentration of each component = 0·2 mg./ml.).

results are set out in Table 5 in order of decreasing nucleic acid content at the end of the first incubation. Inspection shows that, as the nucleic acid content has increased, the rate of accumulation of free glutamic acid has decreased. There is no clear-cut correlation between nucleic acid content and rate of protein synthesis, but this might be masked by the considerable error in the measurement of rates of protein synthesis in some of these examples. It is interesting to observe that the inhibitory action of chloramphenicol on protein synthesis (Gale & Folkes, 1953) during the first incubation does not persist when the cells are washed and resuspended in an amino-acid mixture. There is an increase of approximately 65% in the nucleic acid content of cells incubated with glucose, amino-acids and mixture *P'* in the presence of chloramphenicol; this increase is associated with an increase of 71% in the rate of protein synthesis by the washed cells while their rate of accumulation of free glutamic acid has decreased by 43%.

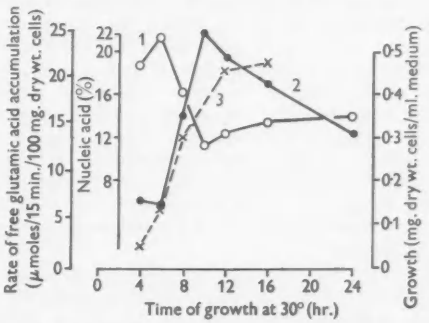
Rate of free glutamic acid accumulation (μmoles/15 min./100 mg. dry wt. cells)
Nucleic acid (%)
Growth (mg. dry wt. cells/ml. medium)
Time of growth at 30° (hr.)
1
2
3

Fig. 6. Variation with age of culture in *Staph. aureus* Duncan of nucleic acid content (curve 1), rate of accumulation of free glutamic acid (curve 2) and growth (curve 3). Conditions as for Fig. 5 with the exception that the suspension was incubated for 15 min. at 37° in buffered salt solution containing 1% glucose and 2·7 μmoles sodium glutamate/ml. for determination of rate of accumulation of free glutamic acid.

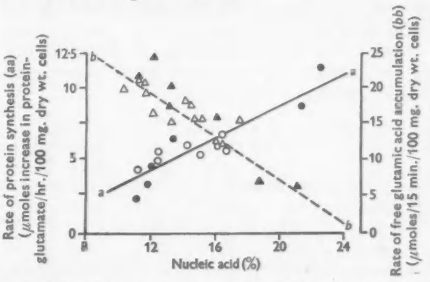
Rate of protein synthesis (aa) (μmoles increase in protein-glutamate/hr./100 mg. dry wt. cells)
Rate of free glutamic acid accumulation (bb) (μmoles/15 min./100 mg. dry wt. cells)
Nucleic acid (%)
a
b

Fig. 7. Correlations between nucleic acid content of *Staph. aureus* Duncan and rate of protein synthesis (*aa*), or rate of accumulation of free glutamic acid (*bb*). *Rate of protein synthesis.* Solid circles (●) represent determinations with cells of differing age of culture as in Fig. 5; open circles (○) determinations with washed suspensions as in Table 5. $A = 0{\cdot}54y - 2{\cdot}02$ where $A$ = rate of increase in protein glutamate in μmoles/100 mg. dry wt. of cells/hr. and $y$ = % nucleic acid content of cells. $n = 14$, $r = +0{\cdot}875$, $P < 0{\cdot}01$. *Rate of free glutamic acid accumulation.* Solid triangles (▲) represent determinations with cells of differing age of culture as in Fig. 6; open triangles (△) determinations with washed suspensions as in Table 5. $B = 37{\cdot}2 - 1{\cdot}5y$ where $B$ = rate of accumulation of free glutamic acid in μmoles/100 mg. dry wt. of cells/15 min. and $y$ = % nucleic acid content of cells. $n = 20$, $r = -0{\cdot}812$, $P < 0{\cdot}01$.

*Variation of nucleic acid content in growing cells.* A large batch of medium was inoculated and samples of culture withdrawn at intervals through growth at 30°. Washed suspensions were made at each stage and, as before, nucleic acid content and rates of

accumulation of free glutamic acid or protein synthesis (in the absence of added purines and pyrimidines) determined. The variations described by Malmgren & Heden (1947) for 'nucleotide' content of the cells were found to hold for the nucleic acid content. Figs. 5 and 6 show the variations obtained in two experiments; in one (Fig. 5) the rate of protein synthesis was determined at each stage and it can be seen that there is a close correlation between that rate and the nucleic acid content at harvesting of the cells; in the other (Fig. 6) it can be seen that the rate of accumulation of free glutamic acid varies inversely as the nucleic acid content.

*Correlations.* Results from the experiments with both washed and growing cells have been plotted and analysed statistically in Fig. 7. A highly significant correlation is found between the nucleic acid content of the cell and its rate of protein synthesis; the values obtained in Table 5 accord well with the correlation obtained, especially when the experimental error is taken into account. An equally significant but negative correlation is obtained between the nucleic acid content of the cell and its ability to take up and concentrate free glutamic acid.

*Discussion.* The discussion of these results is reserved for the end of the following paper (Gale & Folkes, 1953).

## SUMMARY

1. Increase in the protein content of *Staphylococcus aureus* cells takes place if these are incubated in the presence of glucose and a mixture of amino-acids which must include all those essential for growth.

2. Increase in the nucleic acid content of the cells takes place if they are incubated in the presence of glucose and a mixture of purines, pyrimidines and amino-acids. Little or no formation of nucleic acid occurs in the absence of added amino-acids, and optimal synthesis occurs when the amino-acid mixture is also optimal for protein synthesis.

3. The addition of purines and pyrimidines to cells incubated in glucose and amino-acids results in a marked acceleration in the rate of protein synthesis.

4. There is a strong positive correlation between the rate of protein synthesis and the nucleic acid content of the cells, the former falling to zero when the latter has fallen to about 4%.

5. The rate at which cells, incubated with glucose and glutamic acid, accumulate free glutamic acid varies inversely with the nucleic acid content.

The authors are indebted to Dr R. Markham for a gift of cytosine and for advice on chromatographic procedures; to Dr J. Tosic for total nitrogen determinations; to Mr B. Slater for amino-nitrogen determinations and to Miss P. J. Samuels for assistance with the chromatography.

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# The Assimilation of Amino-acids by Bacteria

## 15. ACTIONS OF ANTIBIOTICS ON NUCLEIC ACID AND PROTEIN SYNTHESIS IN *STAPHYLOCOCCUS AUREUS*

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In previous papers of this series, experimental conditions have been described which will enable the following processes to be studied in washed suspensions of *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*): internal accumulation of free glutamic acid (Gale, 1947*a*, *b*), extracellular accumulation of peptides containing glutamic acid (Gale & Van Halteren, 1951), accumulation of combined glutamic acid within the cells (Gale, 1951*b*) and synthesis of protein and nucleic acid (Gale & Folkes, 1953). Aureomycin and chloramphenicol inhibit the increase of cellular combined glutamate at lower concentrations than those necessary to prevent the accumulation of free glutamic acid within the cells, whereas sodium azide, 2:4-dinitrophenol and 8-hydroxyquinoline affect these processes to approximately the same extent (Gale & Paine, 1951). Penicillin and bacitracin have no effect upon the accumulation of free or combined glutamic acid in washed suspensions of cells, but if either of these antibiotics is added to the growth medium an hour before harvesting, the resulting cells are unable to accumulate either free or combined glutamic acid (Gale & Taylor, 1947; Gale & Paine, 1951; Paine, 1951). Hotchkiss (1950) has described a disorganization of protein synthesis by penicillin which results, under the conditions of his experiments, in extracellular accumulation of peptides when *Staph. aureus* is incubated with amino-acid mixtures.

Mitchell & Moyle (1951) followed the nucleic acid content of *Staph. aureus* during normal growth and in the presence of penicillin. During the normal phase of accelerated growth they found that nucleic acid reproduced more rapidly than cell dry weight, suggesting that nucleic acid controlled the rate of cell synthesis. A disturbance of nucleic acid metabolism occurred in the presence of penicillin and was accompanied by an intracellular accumulation of extractable nucleotides. Krampitz & Werkman (1947) and Gros & Macheboeuf (1948*a*, *b*) have previously described inhibitions of nucleic acid metabolism in bacterial cells by high concentrations of penicillin, while Gros, Beljanski & Macheboeuf (1951) have found that penicillin inhibits the breakdown of guanosine by sensitive strains of *Staph. aureus*. Park & Johnson (1949) described the accumulation of labile phosphate compounds in *Staph. aureus* growing in the presence of penicillin, and Park (1952) identified three substances accumulating under these conditions; one of these is a derivative of uridine-5′-pyrophosphate and an *N*-acetylaminosugar, and the other two possess the same basic structure in combination with either L-alanine or a peptide containing L-lysine, D-glutamic acid and DL-alanine.

### METHODS

The organism, growth medium, amino-acid and purine-pyrimidine mixtures (*A*, *P* and *P′*), methods of estimation of free or combined glutamic acid, protein, nucleic acid, purine and pyrimidine bases and rates of fermentation are the same as those described in the preceding paper (Gale & Folkes, 1953).

*Growth inhibition.* Concentrations of antibiotics inhibitory to growth were determined by adding serial dilutions of the drugs to a fully nutrient medium containing 3% tryptic digest of casein, 0·1% Marmite and 1% glucose. Tubes were inoculated with approx. $10^6$ cells/10 ml. medium and growth inspected after 48 hr. incubation at 37°.

### RESULTS

The action of a number of antibiotics and inhibitors has been tested on the following processes in washed suspensions of *Staph. aureus* Duncan: (1) fermentation in the presence of glucose; (2) accumulation of free glutamic acid within the cells when incubation takes place in the presence of glucose and glutamic acid, the latter being the only amino-acid present; (3) protein synthesis when glucose and the complete amino-acid mixture *A* are present; (4) protein and nucleic acid synthesis when the incubation mixture contains glucose and mixtures *A* and *P* or *P′*. In general, it has been found that, except in the case of penicillin, the degree of inhibition of protein synthesis is the same whether mixture *P* is present or not.

*Chloramphenicol.* Fig. 1 shows that protein synthesis is very sensitive to chloramphenicol, 90% inhibition being produced by that concentration necessary to prevent growth of the organism in a complete medium. The processes of fermentation, respiration, free glutamic acid accumulation and nucleic acid synthesis are not inhibited by chloramphenicol except in very high concentrations. Chloramphenicol (30–100 $\mu$g./ml.) produces complete inhibition of protein synthesis and a significant increase in the rate of increase of nucleic acid (see also Table 1). Fig. 2, which shows progress curves for the increase in protein and nucleic acid of washed cells of *Staph. aureus* Duncan, also shows the effect of 30 $\mu$g. chloramphenicol on these processes. Gale & Folkes (1953) found little or no increase in the nucleic acid content of such cells when incubated with glucose and the purine-pyrimidine mixture $P'$ in the absence of amino-acids, but the addition of chloramphenicol gives rise to a significant increase in nucleic acid under these conditions; the stimulation produced by the antibiotic is approximately the same whether the control synthesis is promoted by amino-acids or not. Table 1 shows that the increase in nucleic acid, which occurs in the presence of chloramphenicol and amino-acids, resides in the ribonucleic acid fraction since there is no increase in the thymine content.

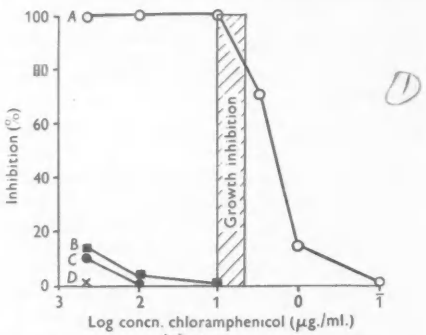


Fig. 1. Actions of chloramphenicol on amino-acid assimilation in *Staph. aureus* Duncan. Cells grown for 16 hr. at 30°; made into washed suspension and tested at final suspension density of 2·0 mg./ml. in the following systems. Curve *A*; rate of increase in protein-glutamate in cells (analyses on TCA precipitates) studied over 1 hr. incubation at 37° in buffered salt solution containing 1% glucose, amino-acid mixture *A* (each amino-acid at concentration of 0·2 mg./ml.) and purine-pyrimidine mixture $P'$ (each component at concentration of 0·02 mg./ml.). Rate of increase in absence of any inhibitor = 12·5 $\mu$moles protein-glutamate/hr./100 mg. dry wt. of cells. Curve *B*; rate of increase of nucleic acid content of cells treated as for curve *A*. Rate of increase in absence of any inhibitor = 6·5 mg. nucleic acid/hr./100 mg. dry wt. of cells. Curve *C*; rate of accumulation of free glutamic acid within the cells studied over 30 min. incubation in buffered salt solution containing 1% glucose and 2·7 $\mu$moles sodium glutamate/ml. Rate of accumulation in absence of any inhibitor = 42 $\mu$moles glutamic acid/hr./100 mg. dry wt. of cells. Curve *D*, rate of fermentation of glucose in bicarbonate buffer at pH 7·2. Rate in absence of any inhibitor = $Q_{CO_2}$, 95. Growth inhibition shows range of drug concentration preventing growth of inoculum of approx. $10^6$ cells/10 ml. complete medium. Temperature, 37° in all cases.

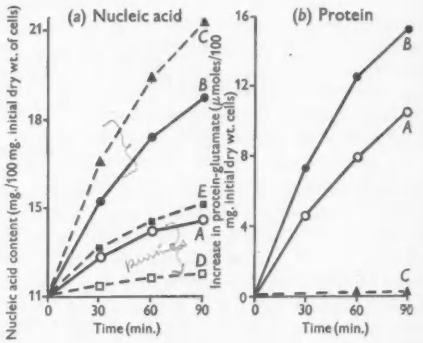


Fig. 2. Effect of chloramphenicol on (*a*) nucleic acid synthesis and (*b*) protein synthesis in *Staph. aureus* Duncan incubated at 37° in buffered salt solution containing 1% glucose and additions as follows. Curve *A*, amino-acid mixture *A*; Curve *B*, amino-acid mixture *A* + purine-pyrimidine mixture $P'$; Curve *C*, as (*B*) + 30 $\mu$g. chloramphenicol/ml.; Curve *D*, mixture $P'$; Curve *E*, as (*D*) + 30 $\mu$g. chloramphenicol/ml. Cells grown for 16 hr. at 30° in 'deficient' medium; made into washed suspension and incubated at final suspension density of 2·0 mg. dry wt. of cells/ml. in above mixtures. Final concentration of each amino-acid = 0·2 mg./ml., of each purine or pyrimidine = 0·02 mg./ml. After incubation, cells precipitated in the cold with 5% TCA. Protein estimated by glutamic acid content of precipitate after acid hydrolysis. Nucleic acid extracted from precipitates with hot 5% TCA and estimated spectrophotometrically.

*Aureomycin and terramycin.* These antibiotics have inhibitory actions similar to those of chloramphenicol except that, at high concentrations, they inhibit the processes of fermentation, accumulation of free glutamic acid and synthesis of nucleic acid (Figs. 3 and 4, Table 2). Fig. 3 shows that protein synthesis is markedly more sensitive to aureomycin than is the accumulation of free glutamic acid within the cells, and that growth is prevented by a concentration of the antibiotic which produces approx. 90% inhibition of the rate of protein synthesis. Concentrations of either antibiotic which inhibit protein synthesis, but do not inhibit nucleic acid synthesis, produce a significant stimulation of the rate of nucleic acid synthesis. The

Table 1. *Composition of nucleic acid fraction of* Staphylococcus aureus *Duncan treated with various antibiotics*

(*A. Staph. aureus* Duncan grown for 6 hr. at 37° with and without the addition of penicillin (10 international units/ml.) to the medium after 4 hr. growth. Cells harvested, washed, precipitated with 5% (w/v) trichloroacetic acid (TCA) in the cold; precipitates washed with cold 5% TCA, then extracted three successive times with 5% TCA at 90° for 10 min. Extracts combined for analysis of bases, etc. *B. Staph. aureus* Duncan grown for 16 hr. at 30° in deficient medium; harvested and made into washed suspension. Cells incubated for 1 hr. in buffered salt solution containing glucose, amino-acid mixture, purine-pyrimidine mixture *P'* and additions as shown below. After incubation, cells washed and nucleic acid extracts prepared as in *A*.)

| | Culture *A* | | Culture *B* | | | | |
|---|---|---|---|---|---|---|---|
| | | | | Additions to incubation medium | | | |
| | Control (% dry wt.) | Penicillin treated (% control) | Initial (% dry wt.) | None | Chloramphenicol (30 μg./ml.) | Penicillin (3000 units/ml.) | Bacitracin (2·5 mg./ml.) |
| | | | | (Results expressed as % initial values) | | | |
| Total nucleic acid | 14·1 | 89 | 10·30 | 126 | 148 | 114 | 110 |
| Adenine | 1·34 | 90 | 0·86 | 125 | 143 | 117 | 107 |
| Guanine | 1·68 | 91 | 1·02 | 126 | 144 | 116 | 115 |
| Cytosine | 0·90 | 92 | 0·66 | 125 | 139 | 116 | 108 |
| Uracil | 0·62 | 90 | 0·535 | 126 | 151 | 115 | 108 |
| Thymine | 0·22 | 90 | 0·25 | 108 | 95 | 110 | 92 |

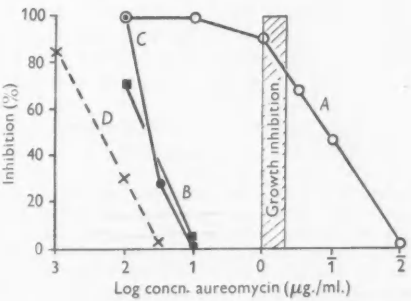


Fig. 3. Actions of aureomycin on amino-acid assimilation in *Staph. aureus* Duncan. Conditions as for Fig. 1.

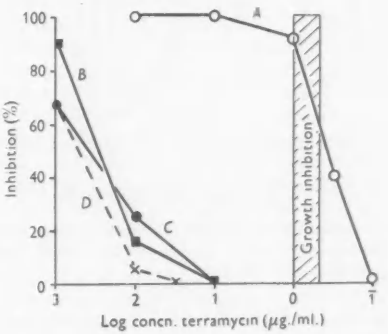


Fig. 4. Actions of terramycin on amino-acid assimilation in *Staph. aureus* Duncan. Conditions as for Fig. 1.

Table 2. *Effect of antibiotics and inhibitors on protein and nucleic acid synthesis, accumulation of free glutamic acid and glucose fermentation in* Staphylococcus aureus

(Conditions as for Fig. 1 using washed suspensions for all tests other than growth.)

| | Concentration which inhibits growth (μg./ml.) | Concentration producing 50% inhibition (μg./ml.) | | | | |
|---|---|---|---|---|---|---|
| | | Protein synthesis | Purine-stimulated protein synthesis | Nucleic acid synthesis | Free glutamic acid accumulation | Glucose fermentation |
| Aureomycin | 0·5–1·0 | 0·2 | 0·2 | 50 | 50 | 200 |
| Terramycin | 0·5–1·0 | 0·4 | — | 300 | 500 | 600 |
| Chloramphenicol | 5–10 | 2·0 | 2·0 | * | * | * |
| Penicillin | 0·05 | * | 200 | 2000 | * | * |
| Bacitracin | 50–100 | 1000 | 1000 | 1000 | * | * |
| Streptomycin | 1–2 | 3000 | — | * | * | * |
| Polymyxin | * | * | * | * | * | * |
| 2:4-Dinitrophenol | Approx. 120 | 60 | — | 60 | 60 | 6000 |
| Sodium azide | Approx. 600 | 320 | — | 320 | 260 | 3200 |

* Little or no inhibition at 1000 μg./ml.

synthesis of nucleic acid has the same sensitivity as the accumulation of free glutamic acid. In general the actions of terramycin are very similar to those of aureomycin except that the former does not differentiate clearly between glucose fermentation and the processes of nucleic acid synthesis or glutamic acid accumulation.

*Uncoupling agents.* In a previous paper of this series (Gale, 1951*a*) it was shown that the drugs, sodium azide and 2:4-dinitrophenol, known to act as uncoupling agents in mitochondrial preparations, inhibit free glutamic acid accumulation at concentrations significantly smaller than those required to inhibit respiration or fermentation. This was confirmed in the present studies, and it was found (Table 2) that protein and nucleic acid syntheses had significantly the same sensitivity to both these drugs as glutamic acid accumulation.

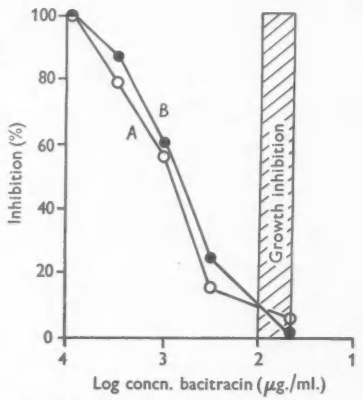


Fig. 5. Inhibition of protein (*A*) and nucleic acid (*B*) syntheses by bacitracin in *Staph. aureus* Duncan. Conditions as for Fig. 1.

*Streptomycin.* Table 2 shows that none of the processes studied in these experiments is inhibited by concentrations of streptomycin of the order of those preventing growth. The synthesis of protein is more sensitive than either free glutamic acid accumulation or nucleic acid synthesis, but 1 mg. streptomycin/ml. produces less than 50% inhibition of the rate of protein synthesis.

*Polymyxin.* Polymyxin in concentrations of 100 μg./ml. had no effect on any of the processes studied here in *Staph. aureus*.

*Bacitracin. Staph. aureus* Duncan is unable to grow if 50–100 μg. bacitracin/ml. are added to the growth medium; concentrations of this order have little effect upon either protein or nucleic acid synthesis by washed cells, whereas concentrations of 2–10 mg. bacitracin/ml. have marked inhibitory actions on both protein and nucleic acid synthesis (Fig. 5), the two processes having significantly the same sensitivity to the antibiotic.

*Penicillin.* It has not been possible to demonstrate, in previous experiments of this series, any action of penicillin even in high concentrations (300 international units/ml.) on the accumulation of free or combined glutamic acid by washed suspensions of *Staph. aureus* Duncan. In view of the reports by Krampitz & Werkman (1947), Gros & Macheboeuf (1948*a*, *b*), Gros *et al.* (1951) and Mitchell & Moyle (1951), it seemed desirable to re-investigate the effects on protein synthesis, especially when such synthesis took place in the presence of added purines and pyrimidines. The results are summarized in Fig. 6: penicillin has little or no effect on the synthesis of protein by washed cells when the incubation mixture is devoid of purines and pyrimidines but, in high concentrations, abolishes the additional synthesis promoted by the addition of mixture *P*. The additional nucleic acid synthesis taking place on the addition of mixture *P* is also decreased by the presence of penicillin, but it can be seen that a concentration of penicillin which completely abolishes the additional protein formation,

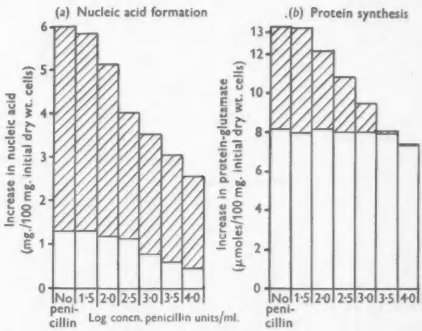


Fig. 6. Effect of penicillin on (*a*) nucleic acid and (*b*) protein synthesis by *Staph. aureus* Duncan in the absence (unshaded portion) and presence (unshaded + shaded) of purine-pyrimidine mixture *P*. Cells grown for 16 hr. at 30° in deficient medium; harvested and made into washed suspension. Cells, at suspension density of 2·0 mg. dry wt./ml. incubated for 1 hr. at 37° in buffered salt solution containing 1% glucose, amino-acid mixture *A* (each amino-acid at final concentration of 0·2 mg./ml.) with and without mixture *P* (each component at 0·02 mg./ml.). After incubation, cells precipitated in the cold with 5% TCA (final concn.). Protein-glutamate determined on portion of precipitate after acid hydrolysis; nucleic acid extracted with hot 5% TCA from remainder of precipitate and estimated spectrophotometrically. Initial values: nucleic acid = 11·2 mg./100 mg. dry wt. of cells; protein-glutamate = 33·5 μmoles/100 mg. dry weight of cells.

does no more than halve the rate of additional nucleic acid synthesis. If there is a metabolic connexion between the additional nucleic acid formation and the additional protein synthesis on the inclusion of mixture *P* in the incubation mixture, it might be that the material formed in the presence of *P* and penicillin (and which is measured simply as the 260 m$\mu$.-absorbing material in the nucleic acid fraction) is not normal nucleic acid. However, it is clear from Table 1 that there has been no gross alteration in the relative proportions of the purine and pyrimidine bases, within experimental error, in the nucleic acid fraction.

## DISCUSSION

The effects of amino-acid and purine-pyrimidine mixtures on increases in the protein and nucleic acid contents of cells clearly suggest a metabolic connexion between the synthesis of protein and nucleic acid (Gale & Folkes, 1953). There is little or no increase in the nucleic acid of the cells unless amino-acids are present in the incubation mixture; if an amino-acid mixture which promotes net protein synthesis is present, then a rapid increase in nucleic acid occurs; single amino-acids or simple mixtures thereof have smaller effects which might be correlated with their ability to promote protein turnover within the cell or otherwise 'spare' protein catabolism. Conversely, the presence of nucleic acid precursors accelerates the synthesis not only of nucleic acid but also of protein, while the addition of penicillin decreases the synthesis of nucleic acid and abolishes the purine-stimulated protein formation. These findings accord well with the various theories that have been put forward during recent years concerning a role of nucleic acid in protein synthesis (reviewed by Chantrenne, 1952). If nucleic acid acts as an organizer or template for protein synthesis, then increased nucleic acid will mean increased protein synthesis, and the reverse relationship may also hold (Caldwell & Hinshelwood, 1950). There are many indications in the literature that penicillin plays some interfering part in nucleic acid metabolism (Krampitz & Werkman, 1947; Gros & Macheboeuf, 1948*a*, *b*; Hotchkiss, 1949; Mitchell & Moyle, 1951; Gros *et al.* 1951; Park, 1952; Hotchkiss, 1952); this might result in an inhibition of nucleic acid synthesis as a whole or of some anabolic process which would lead to formation of an altered nucleic acid. The transformation of sensitive organisms to resistant ones (Hotchkiss, 1952) and the continued formation of adaptive penicillinase after removal of the substrate (Pollock, 1950) would suggest the latter mode of action. No gross changes in the base composition of the nucleic acid formed in the presence of penicillin could be shown in these studies (Table 1), but many alterations may take place in a complex structure such as nucleic acid without resulting in alterations in this type of analysis. If we accept a catalytic role of nucleic acid in protein synthesis, bacitracin would appear to inactivate the nucleic acid already formed in addition to preventing further synthesis of nucleic acid or protein; in this it differs from penicillin.

The action of chloramphenicol, aureomycin and terramycin is to effect a clear-cut inhibition of protein synthesis which is accompanied at the same time by an increase in the rate of nucleic acid formation within the cell. If protein and nucleic acid syntheses pass through a common stage, then this must be followed by splitting away of protein, or its immediate precursor, from the nucleoprotein complex and it may be that this group of antibiotics acts at this point. On the hypothesis of Caldwell & Hinshelwood (1950) such action might well be accompanied by a stimulation of nucleic acid synthesis.

The uncoupling agents, sodium azide and 2:4-dinitrophenol, inhibit the accumulation of free glutamic acid and the syntheses of protein and nucleic acid at concentrations significantly less than those which inhibit fermentation; this differential action may be due to coupled phosphorylations being involved in the more sensitive reactions. Loomis (1950) found that aureomycin at concentrations of 100 $\mu$g./ml. will uncouple oxidative phosphorylation in mitochondrial preparations but that such concentrations are toxic to guinea pigs. Similar concentrations inhibit the accumulation of free glutamic acid by *Staph. aureus* while having little effect on glucose fermentation (see Fig. 3), but protein synthesis is very much more sensitive than either of these processes. Loomis (1950) found that chloramphenicol was not effective as an uncoupling agent in mitochondrial preparations, and it does not effect a differential inhibition of glucose fermentation and glutamic acid accumulation in these studies (Fig. 1).

The results obtained with penicillin in these experiments have been obtained with high concentrations, and it is difficult to interpret the significance of inhibitions produced by concentrations of drug several orders higher than those required to prevent growth of normal inocula. It is possible that inhibition of growth is caused by inhibition of the synthesis of a specific protein or nucleic acid whereas much higher concentrations of the antibiotic are required to inhibit the total protein and nucleic acid syntheses which have been studied here. Gros *et al.* (1951) find that the breakdown of guanylic acid by *Staph. aureus* is inhibited by penicillin but that, whereas 2000–3000 units/ml. are required to inhibit the reaction in washed suspensions, the addition of 3–10 units/ml. to the growth medium 1 hr. before harvesting gives rise to cells whose ability to attack guanylic acid is 10% of that of untreated control cells. They suggest that peni-

cillin penetrates the growing cell more readily than the 'washed resting' cell. It would, however, seem equally possible that the loss of ability to oxidize guanylic acid following growth in the presence of penicillin is a secondary effect following some primary disorganization, an explanation put forward to explain the somewhat similar results obtained for the effect of penicillin on glutamic acid accumulation (Gale & Rodwell, 1949).

Such difficulties of interpretation do not arise in the cases of chloramphenicol, aureomycin and terramycin where the limiting concentration affecting protein synthesis is the same as that affecting growth.

## SUMMARY

1. Chloramphenicol, aureomycin and terramycin inhibit protein synthesis by washed suspensions of *Staphylococcus aureus*, at bactericidal concentrations. Such concentrations simultaneously stimulate nucleic acid synthesis.

2. Bacitracin inhibits protein and nucleic acid synthesis to the same extent. Complete inhibition requires a concentration of bacitracin 100 times that which prevents growth.

3. Penicillin in high concentrations prevents the acceleration of protein synthesis produced by the addition of purines and pyrimidines to the incubation mixture containing amino-acids and glucose.

The authors are indebted to Miss P. J. Samuels for assistance with chromatographic procedures and to Dr T. F. Paine for gifts of aureomycin, streptomycin and bacitracin.

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# The Oxidation of Certain Dicarboxylic Acids by Peroxidase Systems in Presence of Manganese

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It has frequently been suggested that the physiological effects of manganese may be due to its capacity for valency change. Kenten & Mann (1950) have shown that $Mn^{2+}$ is oxidized by peroxidase systems. Such oxidation takes place in plant extracts (Kenten & Mann, 1951) and preliminary results suggest that it also occurs *in vivo* in higher plants. Kenten & Mann (1949) suggested that the manganese oxidation product reacts with plant metabolites whereby the $Mn^{2+}$ is involved in an oxidation-reduction cycle which could explain its effect on plant respiration. As yet, however, there is little evidence as to the nature of the metabolites oxidized.

A number of instances have been recorded where an activating effect of $Mn^{2+}$ on oxidative reactions is

associated with the presence of peroxidase or compounds possessing peroxidatic activity. Thus the rate of oxidation of dihydroxymaleic acid by cytochrome *c* or peroxidase is increased by $Mn^{2+}$ (Theorell & Swedin, 1939; Swedin & Theorell, 1940). It is well known that the spontaneous and enzyme-catalysed decarboxylations of oxaloacetate and oxalosuccinate are accelerated by a variety of polyvalent cations; $Mn^{2+}$ is usually the most effective cation for the enzyme-catalysed reactions (Krebs, 1942; Speck, 1949; Kornberg, Ochoa & Mehler, 1948; Gollub & Vennesland, 1947; Vennesland, Ceithaml & Gollub, 1947). In some cases an oxidative decarboxylation of oxaloacetate and oxalosuccinate has been found when $Mn^{2+}$ has been used to activate the enzyme preparations from plant tissues (Vennesland & Felsher, 1946; Ceithaml & Vennesland, 1949). Vennesland & Evans (1944) found that oxaloacetic acid was oxidatively decarboxylated to malonic acid by a preparation from heart muscle in presence of $Mn^{2+}$. Vennesland, Evans & Francis (1946) found that the active factor in such preparations was metmyoglobin and pointed out the similarity between the reaction and the oxidation of dihydroxymaleic acid catalysed by cytochrome *c* and $Mn^{2+}$. Since it is now known that peroxidase systems oxidize $Mn^{2+}$ it appeared possible that the effect of $Mn^{2+}$ in these systems was due to its oxidation through peroxidase or the peroxidatic action of metmyoglobin and cytochrome *c*. The present work was undertaken to investigate this possibility.

## MATERIALS AND METHODS

*Peroxidase and catalase preparations.* These were as described by Kenten & Mann (1952).

*Plant saps.* The plant material was washed free from soil, ground in a mortar with sand and squeezed through madapollam.

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*Dihydroxymaleate solutions.* These were prepared immediately before use by dissolving dihydroxymaleic acid in 0·05M-acetate buffer, pH 5, and adjusting to pH 5 with N-NaOH.

*Dihydroxymaleic acid.* This was prepared by the action of $H_2O_2$ on tartaric acid according to the method of Fenton (1894). The recrystallized product decomposed at 152° (uncorr.). Fenton (1894) gives the decomposition temperature as about 155°.

*Manometric measurements.* These were made in the Warburg apparatus at 25°. The volume of the reaction mixtures was 3 ml. $O_2$ uptakes were measured directly with KOH in the centre cups. $CO_2$ outputs were measured by Warburg's direct method (Dixon, 1943).

## EXPERIMENTAL AND RESULTS

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Kenten & Mann (1952) showed that manganese oxidation could be brought about by coupling enzyme systems producing $H_2O_2$ with peroxidase systems. An increased $O_2$ uptake was obtained when oxalate was added to such systems in presence but not in absence of $Mn^{2+}$. This was attributed to a manganese oxidation-reduction cycle, but the results obtained suggested that reactions were proceeding in addition to the coupled oxidation of the manganese by the enzyme system followed by reduction of the oxidized manganese by the oxalate. Launer (1933) showed that when $Mn^{3+}$ was added to oxalate solutions the $CO_2$ output in air was considerably greater than that required for complete reduction of the $Mn^{3+}$ to $Mn^{2+}$ by the oxalate. Evidence was obtained that this was due to re-oxidation of the $Mn^{2+}$ accompanied by $H_2O_2$ formation. In the present work experiments were made to investigate whether such $H_2O_2$ formation affected the interpretation of the results of Kenten & Mann (1952). It was found that, while reaction mixtures containing oxalate and $Mn^{2+}$ alone showed no $O_2$ uptake, the addition of peroxidase together with *p*-cresol to such reaction mixtures caused an $O_2$ uptake after an initial lag period. The rate of $O_2$ uptake depended on the concentration of $Mn^{2+}$. A typical set of curves is shown in Fig. 1. Reaction mixtures consisted of 100 $\mu$g. peroxidase of purpurogallin number (P.Z.) 250 (Willstätter & Stoll, 1918), 0·3 ml. 0·001M-*p*-cresol and 0·5 ml. 0·1M-sodium oxalate, pH 5, with 1 ml. 0·1M-acetate buffer, pH 5. Manganese sulphate (0·2 ml. 0·1M or 0·01M) was added from the side arm after equilibration. Control experiments were made in which peroxidase, *p*-cresol or $Mn^{2+}$ were omitted.

The fact that the $O_2$ uptake is dependent on the presence of peroxidase and *p*-cresol suggests that $H_2O_2$ formation and manganese oxidation are involved in the reaction mechanism. With the higher $Mn^{2+}$ concentration a faint pink colour was present in the reaction mixture at the end of the lag period. This colour became more intense during the period of rapid $O_2$ uptake and then decreased in intensity. The pink colour is probably due to the presence of a manganioxalate complex such as those described by Cartledge & Ericks (1936). The lag period could be reduced by adding small amounts of $H_2O_2$ (0·1 ml. 0·005M) from the side arm, a fact which supports the suggestion that $H_2O_2$ is involved in the reaction. As would be expected, the added $H_2O_2$ caused oxidation of the $Mn^{2+}$ and the reaction mixture rapidly became pink owing to the formation of mangani-

cillin penetrates the growing cell more readily than the 'washed resting' cell. It would, however, seem equally possible that the loss of ability to oxidize guanylic acid following growth in the presence of penicillin is a secondary effect following some primary disorganization, an explanation put forward to explain the somewhat similar results obtained for the effect of penicillin on glutamic acid accumulation (Gale & Rodwell, 1949).

Such difficulties of interpretation do not arise in the cases of chloramphenicol, aureomycin and terramycin where the limiting concentration affecting protein synthesis is the same as that affecting growth.

## SUMMARY

1. Chloramphenicol, aureomycin and terramycin inhibit protein synthesis by washed suspensions of *Staphylococcus aureus*, at bactericidal concentrations. Such concentrations simultaneously stimulate nucleic acid synthesis.

2. Bacitracin inhibits protein and nucleic acid synthesis to the same extent. Complete inhibition requires a concentration of bacitracin 100 times that which prevents growth.

3. Penicillin in high concentrations prevents the acceleration of protein synthesis produced by the addition of purines and pyrimidines to the incubation mixture containing amino-acids and glucose.

The authors are indebted to Miss P. J. Samuels for assistance with chromatographic procedures and to Dr T. F. Paine for gifts of aureomycin, streptomycin and bacitracin.

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# The Oxidation of Certain Dicarboxylic Acids by Peroxidase Systems in Presence of Manganese

By R. H. KENTEN and P. J. G. MANN
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It has frequently been suggested that the physiological effects of manganese may be due to its capacity for valency change. Kenten & Mann (1950) have shown that $Mn^{2+}$ is oxidized by peroxidase systems. Such oxidation takes place in plant extracts (Kenten & Mann, 1951) and preliminary results suggest that it also occurs *in vivo* in higher plants. Kenten & Mann (1949) suggested that the manganese oxidation product reacts with plant metabolites whereby the $Mn^{2+}$ is involved in an oxidation-reduction cycle which could explain its effect on plant respiration. As yet, however, there is little evidence as to the nature of the metabolites oxidized.

A number of instances have been recorded where an activating effect of $Mn^{2+}$ on oxidative reactions is

associated with the presence of peroxidase or compounds possessing peroxidatic activity. Thus the rate of oxidation of dihydroxymaleic acid by cytochrome *c* or peroxidase is increased by $Mn^{2+}$ (Theorell & Swedin, 1939; Swedin & Theorell, 1940). It is well known that the spontaneous and enzyme-catalysed decarboxylations of oxaloacetate and oxalosuccinate are accelerated by a variety of polyvalent cations; $Mn^{2+}$ is usually the most effective cation for the enzyme-catalysed reactions (Krebs, 1942; Speck, 1949; Kornberg, Ochoa & Mehler, 1948; Gollub & Vennesland, 1947; Vennesland, Ceithaml & Gollub, 1947). In some cases an oxidative decarboxylation of oxaloacetate and oxalosuccinate has been found when $Mn^{2+}$ has been used to activate the enzyme preparations from plant tissues (Vennesland & Felsher, 1946; Ceithaml & Vennesland, 1949). Vennesland & Evans (1944) found that oxaloacetic acid was oxidatively decarboxylated to malonic acid by a preparation from heart muscle in presence of $Mn^{2+}$. Vennesland, Evans & Francis (1946) found that the active factor in such preparations was metmyoglobin and pointed out the similarity between the reaction and the oxidation of dihydroxymaleic acid catalysed by cytochrome *c* and $Mn^{2+}$. Since it is now known that peroxidase systems oxidize $Mn^{2+}$ it appeared possible that the effect of $Mn^{2+}$ in these systems was due to its oxidation through peroxidase or the peroxidatic action of metmyoglobin and cytochrome *c*. The present work was undertaken to investigate this possibility.

## MATERIALS AND METHODS

*Peroxidase and catalase preparations.* These were as described by Kenten & Mann (1952).

*Plant saps.* The plant material was washed free from soil, ground in a mortar with sand and squeezed through madapollam.

*Oxaloacetate and dihydroxytartrate solutions.* These were prepared immediately before use from oxaloacetic and dihydroxytartaric acids and adjusted to the required pH with N-NaOH.

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## EXPERIMENTAL AND RESULTS

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Kenten & Mann (1952) showed that manganese oxidation could be brought about by coupling enzyme systems producing $H_2O_2$ with peroxidase systems. An increased $O_2$ uptake was obtained when oxalate was added to such systems in presence but not in absence of $Mn^{2+}$. This was attributed to a manganese oxidation-reduction cycle, but the results obtained suggested that reactions were proceeding in addition to the coupled oxidation of the manganese by the enzyme system followed by reduction of the oxidized manganese by the oxalate. Launer (1933) showed that when $Mn^{3+}$ was added to oxalate solutions the $CO_2$ output in air was considerably greater than that required for complete reduction of the $Mn^{3+}$ to $Mn^{2+}$ by the oxalate. Evidence was obtained that this was due to re-oxidation of the $Mn^{2+}$ accompanied by $H_2O_2$ formation. In the present work experiments were made to investigate whether such $H_2O_2$ formation affected the interpretation of the results of Kenten & Mann (1952). It was found that, while reaction mixtures containing oxalate and $Mn^{2+}$ alone showed no $O_2$ uptake, the addition of peroxidase together with *p*-cresol to such reaction mixtures caused an $O_2$ uptake after an initial lag period. The rate of $O_2$ uptake depended on the concentration of $Mn^{2+}$. A typical set of curves is shown in Fig. 1. Reaction mixtures consisted of 100 $\mu$g. peroxidase of purpurogallin number (P.Z.) 250 (Willstätter & Stoll, 1918), 0·3 ml. 0·001 M-*p*-cresol and 0·5 ml. 0·1 M-sodium oxalate, pH 5, with 1 ml. 0·1 M-acetate buffer, pH 5. Manganese sulphate (0·2 ml. 0·1 M or 0·01 M) was added from the side arm after equilibration. Control experiments were made in which peroxidase, *p*-cresol or $Mn^{2+}$ were omitted.

The fact that the $O_2$ uptake is dependent on the presence of peroxidase and *p*-cresol suggests that $H_2O_2$ formation and manganese oxidation are involved in the reaction mechanism. With the higher $Mn^{2+}$ concentration a faint pink colour was present in the reaction mixture at the end of the lag period. This colour became more intense during the period of rapid $O_2$ uptake and then decreased in intensity. The pink colour is probably due to the presence of a manganioxalate complex such as those described by Cartledge & Ericks (1936). The lag period could be reduced by adding small amounts of $H_2O_2$ (0·1 ml. 0·005 M) from the side arm, a fact which supports the suggestion that $H_2O_2$ is involved in the reaction. As would be expected, the added $H_2O_2$ caused oxidation of the $Mn^{2+}$ and the reaction mixture rapidly became pink owing to the formation of mangani-

oxalate. It appears, therefore, that the lag period may be due to the slow build-up of manganioxalate. Once sufficient of this is present the $O_2$ uptake and manganioxalate formation proceed rapidly. Manganioxalate could be rapidly formed in the reaction mixtures by adding small amounts of hydrated $MnO_2$ (e.g. 50 $\mu$g. $MnO_2$) which were reduced by the $Mn^{2+}$ present to the $Mn^{3+}$ state. Such additions of $MnO_2$ also reduced the lag period.

The $O_2$ uptake was accompanied with a $CO_2$ output and at the end of the experimental period R.Q.'s of about 3·5 were obtained. It is therefore clear that the greater part of the observed $O_2$ uptake represents oxidation of the oxalate.

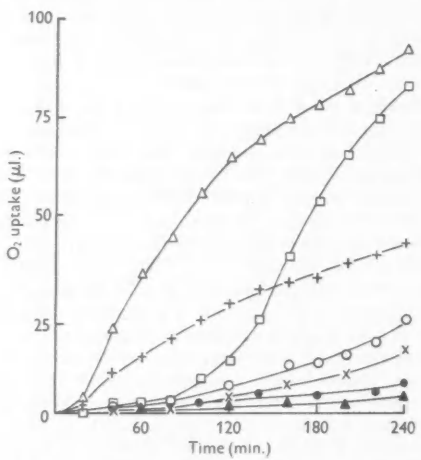


Fig. 1. Catalysis of the oxidation of oxalate by peroxidase, $Mn^{2+}$ and *p*-cresol. Complete system with 1100 $\mu$g. $Mn^{2+}$: □—□, no $H_2O_2$; △—△, primed with 0·1 ml. 0·005 M-$H_2O_2$. Complete system with 110 $\mu$g. $Mn^{2+}$: ○—○, no $H_2O_2$; +—+, primed with 0·1 ml. 0·005 M-$H_2O_2$. Controls with 1100 $\mu$g. $Mn^{2+}$: ▲—▲, peroxidase omitted; ×—×, *p*-cresol omitted; ●—●, $Mn^{2+}$ omitted.

It appeared unlikely that the oxalate reaction could be of physiological importance owing to the relatively high concentrations of $Mn^{2+}$ required. Since it was improbable that the reaction was specific for oxalate a variety of carboxylic acids were tested under similar conditions. Negative results were obtained with citric, aconitic, tartaric, malic, acetoacetic and α-ketoglutaric acids in manometric experiments though, in some cases, the reaction mixtures gave positive colorimetric tests with benzidine suggesting the presence of $H_2O_2$ or of oxidized manganese. In manometric experiments positive results were obtained with oxaloacetic, ketomalonic and dihydroxytartaric acids.

*Oxaloacetate*

With oxaloacetate it was found that the reaction proceeded rapidly at much lower concentrations of $Mn^{2+}$ than with oxalate. A typical set of results is illustrated in Fig. 2. Reaction mixtures were made up as for the experiments of Fig. 1 except that the oxalate was replaced by oxaloacetate (3 mg. oxaloacetic acid) and 0·05, 0·1 and 0·2 ml. 0·01 M-$MnSO_4$ were added from the side arms after equilibration. The lag period was about 15 min., instead of 80 min. in the case of oxalate, after which a

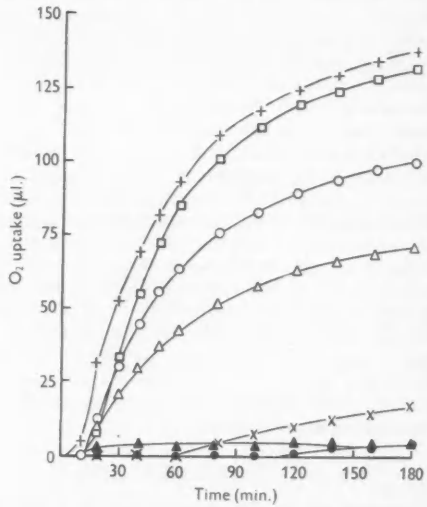


Fig. 2. Catalysis of the oxidation of oxaloacetate by peroxidase, $Mn^{2+}$ and *p*-cresol. Complete system: □—□, 110 $\mu$g. $Mn^{2+}$; ○—○, 55 $\mu$g. $Mn^{2+}$; △—△, 27·5 $\mu$g. $Mn^{2+}$; +—+, 110 $\mu$g. $Mn^{2+}$ primed with 0·1 ml. 0·005 M-$H_2O_2$. Controls with 110 $\mu$g. $Mn^{2+}$: ●—●, peroxidase omitted; ×—×, *p*-cresol omitted; ▲—▲, $Mn^{2+}$ omitted.

comparatively rapid $O_2$ uptake took place, the rate and total uptake increasing with the amount of $Mn^{2+}$ added. The lag period was shortened by addition of 0·1 ml. 0·005 M-$H_2O_2$. The uptake depended on the presence of both peroxidase and *p*-cresol and was far greater than that required for the oxidation of the $Mn^{2+}$ added. The effect of the $Mn^{2+}$ was therefore to catalyse the oxidation of the oxaloacetate. No visual evidence was obtained during the experiment of the accumulation of manganic complexes. But on subsequent addition of benzidine an immediately positive test was obtained only with the complete reaction mixture. This suggests that the positive test was due to oxidized manganese rather than $H_2O_2$. The $O_2$

uptake was accompanied by an increased $CO_2$ output (Fig. 3). Oxaloacetate alone undergoes spontaneous decarboxylation under the experimental conditions. The rate of $CO_2$ output was increased by $Mn^{2+}$ alone (Krebs, 1942; Speck, 1949) but much more markedly by $Mn^{2+}$ where both peroxidase and *p*-cresol were also present. Peroxidase and *p*-cresol alone, in the absence of $Mn^{2+}$, did not affect the spontaneous decarboxylation of oxaloacetate.

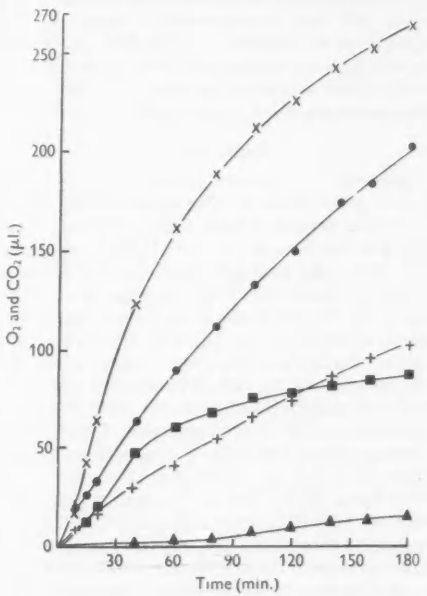


Fig. 3. $CO_2$ output and $O_2$ uptake during catalysis of the oxidation of oxaloacetate by peroxidase, $Mn^{2+}$ and *p*-cresol. $CO_2$ outputs: ×—×, complete system; ●—●, peroxidase omitted; +—+, $Mn^{2+}$ omitted. $O_2$ uptakes: ■—■, complete system; ▲—▲, peroxidase omitted.

*Ketomalonate*

The reaction with ketomalonate was studied in some detail since it proceeds very readily and is not complicated by loss of substrate by spontaneous decarboxylation.

*Effect of variation in* $Mn^{2+}$ *concentration on* $O_2$ *uptake.* This is shown by the typical set of curves of Fig. 4. Reaction mixtures were as for Fig. 2, except that oxaloacetate was replaced by ketomalonate (3 mg. disodium ketomalonate) and amounts of $MnSO_4$ varying from 0·1 ml. 0·001 M to 0·1 ml. 0·01 M were added from the side arms. With the highest amount of $Mn^{2+}$ added (110 µg.) there was a lag period of about 40 min. The lag period decreased with decrease in $Mn^{2+}$ addition. While the length of the lag period varied in different experiments its dependence on the concentration of $Mn^{2+}$ was always observed. The lag period with 110 µg. $Mn^{2+}$ was almost completely eliminated by adding 0·1 ml. 0·005 M-$H_2O_2$ to the reaction mixture. In contrast to the results with oxaloacetate it was found that the oxidation of ketomalonate proceeded, though at a much reduced rate, when *p*-cresol was omitted from the reaction mixtures. At the end of the experiment positive benzidine tests were obtained in the complete reaction mixture and also where *p*-cresol was omitted. The $O_2$ uptake was accompanied by a $CO_2$ output as shown by the results of Fig. 5. Under the conditions used ketomalonate does not undergo

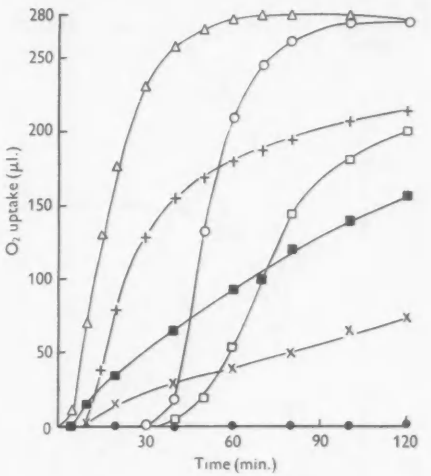


Fig. 4. Catalysis of the oxidation of ketomalonate by peroxidase, $Mn^{2+}$ and *p*-cresol. Complete system: ○—○, 110 µg. $Mn^{2+}$; +—+, 27·5 µg. $Mn^{2+}$; ×—×, 5·5 µg. $Mn^{2+}$; △—△, 110 µg. $Mn^{2+}$ primed with 0·1 ml. of 0·005 M-$H_2O_2$. Controls: □—□, 110 µg. $Mn^{2+}$, *p*-cresol omitted; ●—●, $Mn^{2+}$ omitted, primed with 0·1 ml. 0·005 M-$H_2O_2$.

spontaneous decarboxylation and the correlation of the $CO_2$ output with the $O_2$ uptake was therefore clearer than with oxaloacetate.

*Effect of variation of peroxidase concentration and addition of catalase.* Reaction mixtures to test the effect of variation in peroxidase concentration were as for Fig. 4 except that 0·1 ml. of 0·01 M-$MnSO_4$ (55 µg. $Mn^{2+}$) was present throughout and varying amounts of peroxidase (0–100 µg. of P.Z. 250) were added. A measurable $O_2$ uptake was obtained in presence of 2 µg. peroxidase and the maximum velocity was reached at about 20 µg. Further increase in peroxidase up to 100 µg. produced little change in this velocity. At the lower concentrations of peroxidase little or no $O_2$ uptake was observed

when the *p*-cresol was omitted. The $O_2$ uptake was decreased in rate but not completely eliminated by heat treatment (15 min. in boiling-water bath) of the peroxidase. The effect of this heat treatment was greater with the lower amounts of peroxidase. Catalase prolonged the lag period but did not completely inhibit the oxidation under the conditions used. Thus in one experiment where the reaction was catalysed by 10 $\mu$g. peroxidase (P.Z. 250) the lag period was increased by 10 and 40 min. by 0·1 and 1·0 mg. catalase ($k_1' = 2{\cdot}21 \times 10^6$ M$^{-1}$ sec.$^{-1}$; Chance & Herbert, 1950) respectively.

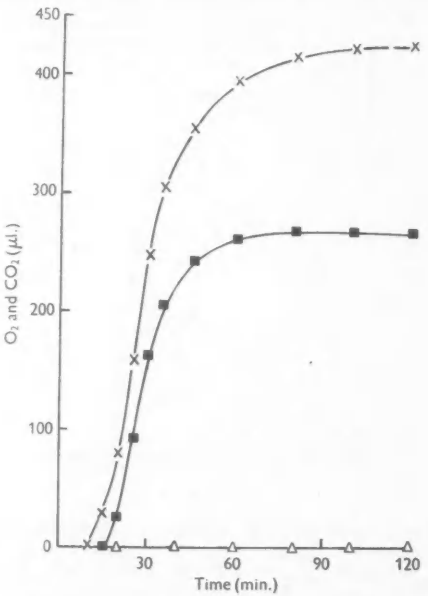


Fig. 5. $CO_2$ output and $O_2$ uptake during catalysis of the oxidation of ketomalonate by peroxidase, $Mn^{2+}$ and *p*-cresol. $CO_2$ outputs: × — ×, complete system; △—△, peroxidase omitted. $O_2$ uptakes: ■—■, complete system; △—△, peroxidase omitted.

*Effect of varying ketomalonate concentrations.* This was studied in reaction mixtures as for Fig. 4 except that 20 $\mu$g. peroxidase of P.Z. 250 were used and the additions of ketomalonate varied between 0·3 and 3·0 mg. The reaction proceeded readily at all the concentrations of ketomalonate used and the total uptakes were roughly proportional to the amounts present. The lag period decreased with decreasing concentration of ketomalonate from about 10 min. with 1 mg./ml. to 2–3 min. with 0·1 mg./ml. For this reason it is difficult to correlate the initial rate of $O_2$ uptake with ketomalonate concentration.

*Effect of addition of metallic ions other than* $Mn^{2+}$. The effect on the oxidative decarboxylation of oxaloacetate and ketomalonate of substituting $Co^{2+}$, $Zn^{2+}$, $Ni^{2+}$, $Al^{3+}$, $Fe^{2+}$ and $Fe^{3+}$ for $Mn^{2+}$ was investigated under the conditions of Fig. 4. Additions of these ions were made at two levels to give final concentrations of 0·066 and 0·0066 M. No appreciable $O_2$ uptake was observed except with $Fe^{2+}$ at the higher concentration. This uptake was unaffected by the presence of peroxidase and *p*-cresol, and was approximately equal to that required for the oxidation of the $Fe^{2+}$ to $Fe^{3+}$. It was therefore concluded that the $O_2$ uptake was due solely to this oxidation and that the oxidation of diketomalonate was not catalysed.

*Dihydroxytartrate*

Each reaction mixture contained 3 mg. sodium dihydroxytartrate in 0·033 M-acetate buffer at pH 5 and, where present, 0·4 ml. 0·001 M-$MnSO_4$, 0·3 ml. 0·001 M-*p*-cresol and 10 or 100 $\mu$g. peroxidase (P.Z. 250). At this pH solutions of dihydroxytartrate undergo rapid spontaneous decarboxylation. The decarboxylation is accompanied by a comparatively slow $O_2$ uptake. The rate of $CO_2$ output was increased by $Mn^{2+}$ though little effect was produced on the rate of $O_2$ uptake. Peroxidase alone had little or no effect on the rate of $O_2$ uptake in absence of $Mn^{2+}$ but in presence of $Mn^{2+}$ caused a rapid uptake. The rate of this uptake was only clearly increased by *p*-cresol at the lower level of peroxidase. Here, during the earlier part of the reaction period, *p*-cresol produced increases of 50–100%. Owing to the rapid spontaneous decarboxylation of the dihydroxytartrate solutions it is not clear how far the oxidation observed is that of dihydroxytartrate or its decarboxylation product.

*Dihydroxymaleate*

Under the conditions used with oxaloacetate, ketomalonate and dihydroxytartrate the oxidation of dihydroxymaleate is rapidly catalysed by peroxidase in presence of $Mn^{2+}$ and the rate is further increased when *p*-cresol also is present. But the results are difficult to interpret since solutions of dihydroxymaleate alone show a small $O_2$ uptake, the rate of which is increased by peroxidase and still further by peroxidase together with *p*-cresol. Also, $Mn^{2+}$ alone causes a large increase in the rate of $O_2$ uptake. A clearer picture is obtained if the reactions are carried out in presence of catalase which markedly reduces the increased rate of $O_2$ uptake produced by $Mn^{2+}$ alone. The results of an experiment in presence of catalase are shown in Fig. 6. Reaction mixtures consisted of 2 mg. dihydroxymaleate in 0·66 M-acetate buffer at pH 5, 0·1 mg. catalase, 0·1 ml. 0·001 M-$MnSO_4$ (5·5 $\mu$g. $Mn^{2+}$) and 0·3 ml. 0·001 M-*p*-cresol. The $Mn^{2+}$ and per-

oxidase were added from the side arms after equilibration. Simultaneous addition of $Mn^{2+}$ and peroxidase produced an increase in the initial rate of $O_2$ uptake much greater than the sum of the increases in rate produced by these factors separately. *p*-Cresol, where present in addition to $Mn^{2+}$ and peroxidase, produced a further increase in rate which was greater than the increase produced by *p*-cresol with peroxidase alone. At the end of the experiment all the reaction mixtures gave negative coloration was discharged by hydrazine with gas evolution and was presumable due to manganipyrophosphate (Kenten & Mann, 1949).

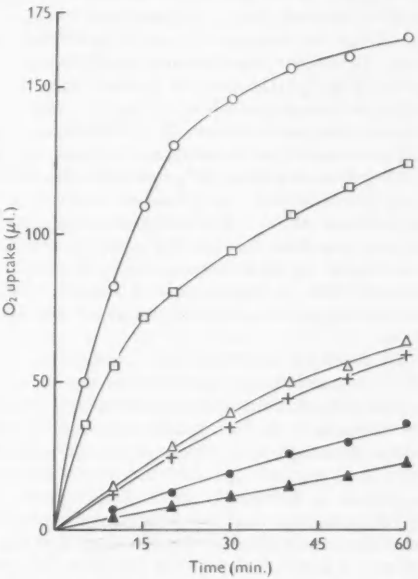


Fig. 6. Catalysis of the oxidation of dihydroxymaleate by peroxidase $Mn^{2+}$ and *p*-cresol. ○—○, complete system; □—□, *p*-cresol omitted; △—△, peroxidase and *p*-cresol omitted; +—+, $Mn^{2+}$ omitted; ●—●, $Mn^{2+}$ and *p*-cresol omitted; ▲—▲, peroxidase, $Mn^{2+}$ and *p*-cresol omitted.

*Oxidation of oxaloacetate and ketomalonate by pea-seedling sap with added* $Mn^{2+}$. Kenten & Mann (1952) have shown that $Mn^{2+}$ is oxidized by saps from many plants by the peroxidase systems present. These

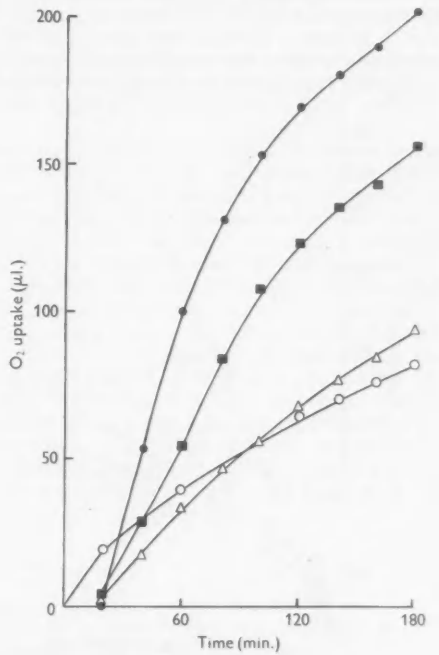


Fig. 7. Oxidation of oxaloacetate by pea-seedling sap in presence of $Mn^{2+}$. ○—○, sap alone or sap + 110 μg. $Mn^{2+}$; △—△, sap and oxaloacetate; ■—■, sap and oxaloacetate + 27·5 μg. $Mn^{2+}$; ●—●, sap and oxaloacetate + 110 μg. $Mn^{2+}$.

tests with benzidine showing that no manganese oxidation product had accumulated. But in pyrophosphate media using relatively high concentrations of $MnSO_4$ colorimetric evidence was obtained of the accumulation of manganipyrophosphate in reaction mixtures containing, in addition, dihydroxymaleate, peroxidase and *p*-cresol. The reaction mixtures were made up in beakers and consisted of 0·5 ml. 0·2 M-pyrophosphate buffer at pH 7 containing 0·2 mg. dihydroxymaleic acid and, where present, 0·2 ml. 0·1 M-$MnSO_4$, 100 μg. peroxidase of P.Z. 250 and 0·3 ml. 0·001 M-*p*-cresol. Water was added to give final volumes of 3 ml. A pink coloration appeared in the complete reaction mixture after about 15 min. at room temperature. This pink

saps with added $Mn^{2+}$ would therefore be expected to oxidize oxaloacetate and ketomalonate. Such oxidation could readily be demonstrated with pea-seedling sap though negative results were obtained with tobacco-leaf sap. Results with pea-seedling sap are illustrated by the curves of Figs. 7 and 8. In these experiments reaction mixtures consisted of 0·5 ml. sap from 7- to 8-day-old pea seedlings and 1 ml. 0·1 M-acetate buffer at pH 5 with additions of $Mn^{2+}$ varying from 0·1 ml. of 0·001 M to 0·2 ml. of 0·01 M-$MnSO_4$. Oxaloacetic acid (3 mg.) or disodium ketomalonate (3 mg.) were added from the side arms after equilibration. A slight output was observed when the oxaloacetate was added and the subsequent apparent $O_2$ uptake was very slow over

the following 15–20 min. After this time oxaloacetate had little effect on the $O_2$ uptake of the sap in absence of added $Mn^{2+}$, but in presence of added $Mn^{2+}$ a markedly increased $O_2$ uptake succeeded the lag period. The rate of this uptake depended on the amount of $Mn^{2+}$ added. In presence of ketomalonate no lag period was observed and the rate of $O_2$ uptake was clearly increased by additions of $MnSO_4$ as low as 0·1 ml. of 0·001 M (5·5 $\mu$g. $Mn^{2+}$). The $O_2$ uptake in presence and absence of ketomalonate and $Mn^{2+}$ was completely eliminated by heating the sap (15 min. in a boiling-water bath).

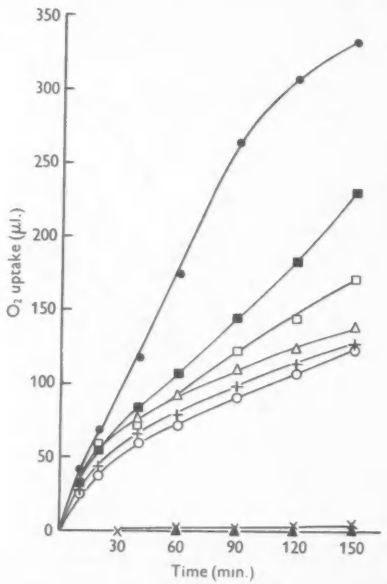


Fig. 8. Oxidation of ketomalonate by pea-seedling sap in presence of $Mn^{2+}$. +—+, sap alone; △—△, sap + ketomalonate; ○—○, sap + 22 $\mu$g. $Mn^{2+}$; □—□, sap + ketomalonate + 5·5 $\mu$g. $Mn^{2+}$; ■—■, sap + ketomalonate + 11 $\mu$g. $Mn^{2+}$; ●—●, sap + ketomalonate + 22 $\mu$g. $Mn^{2+}$. ▲—▲, boiled sap + ketomalonate; ×—×, boiled sap + ketomalonate + 22 $\mu$g. $Mn^{2+}$.

## DISCUSSION

The results of the present work show that $Mn^{2+}$ in presence of peroxidase or peroxidase systems can catalyse the oxidation of certain dicarboxylic acids by $O_2$. At the concentrations of peroxidase used, a peroxidase substrate such as *p*-cresol is necessary for the oxidation of oxalate and oxaloacetate. It has already been shown that peroxidase in presence of *p*-cresol catalyses the oxidation of $Mn^{2+}$ by $H_2O_2$ (Kenten & Mann, 1950). It is therefore reasonable to suggest that the observed oxidation of these two acids is dependent on manganese oxidation. With oxalate, colorimetric evidence suggests the accumulation of manganioxalate during the early stages of rapid oxidation. With oxaloacetate, accumulation of manganic complexes is not apparent, but the reaction mixtures containing $Mn^{2+}$ give strongly positive tests with benzidine suggesting the presence of $H_2O_2$ or of oxidized manganese. The reactions depend on the presence of peroxidase and the lag period can be shortened by adding $H_2O_2$ or increased by catalase. Hydrogen peroxide is therefore presumably formed during the reactions. It is suggested that the mangano-oxalate autoxidizes very slowly under the experimental conditions giving traces of $H_2O_2$. This results in the formation of manganic manganese either during the autoxidation or in the peroxidase-catalysed oxidation of the mangano-oxalate by the $H_2O_2$ so produced. Launer (1933) has shown that $H_2O_2$ is formed during the oxidation of oxalate by manganic manganese in the presence of $O_2$. Once manganic manganese is formed, therefore, further $H_2O_2$ will be produced presumably by the mechanism postulated by Launer (1933). In agreement with this is the finding that the lag period can be shortened by addition of $MnO_2$.

The dependence of the oxidation of ketomalonate, dihydroxytartrate and dihydroxymaleate on the presence of peroxidase substrates such as *p*-cresol is less marked. Thus the oxidation of ketomalonate proceeds, though at a comparatively slow rate, in absence of *p*-cresol provided the peroxidase concentration is sufficiently high. The oxidation of dihydroxytartrate and dihydroxymaleate proceeds at all the concentrations of peroxidase used in the absence of *p*-cresol, though the addition of *p*-cresol increases the rate of oxidation at low peroxidase levels. It is possible that the oxidation of mangano complexes of these acids is directly catalysed by peroxidase. In this connexion it is of interest that peroxidase directly catalyses the oxidation of ferrocyanide, though at a much lower rate than in the presence of *p*-cresol (Kenten & Mann, 1951). The accumulation of mangani complexes of ketomalonate and dihydroxytartrate was not apparent during the oxidations, but positive benzidine tests were obtained during the oxidation of ketomalonate. It should be pointed out that these acids reduce $MnO_2$ much more rapidly than oxalic acid does under the experimental conditions. When the oxidation of dihydroxymaleate by the system peroxidase, $Mn^{2+}$ and *p*-cresol was carried out in pyrophosphate buffer at pH 7, manganipyrophosphate accumulated. The oxidation of dihydroxymaleate differs from that of other compounds used in that it is catalysed by addition of peroxidase alone or of $Mn^{2+}$ alone (Szent-Gyorgyi, 1939; Swedin & Thorell, 1940).

On the basis of these results it is suggested that the previously reported oxidations of dihydroxymaleate and oxaloacetate dependent on the presence of both $Mn^{2+}$ and peroxidase or compounds with peroxidatic activity (Theorell & Swedin, 1939; Swedin & Theorell, 1940; Vennesland *et al.* 1946) involves the oxidation of $Mn^{2+}$. Likewise, it is suggested that the reported oxidation of oxaloacetate and oxalosuccinate by plant-tissue preparation in presence of $Mn^{2+}$ (Vennesland & Felsher, 1946; Ceithaml & Vennesland, 1949) is due to the presence of peroxidase and depends on the oxidation of $Mn^{2+}$.

It is well known since the early work of Thurlow (1925) and Harrison & Thurlow (1926) that peroxidase can catalyse the oxidation of its substrates with $H_2O_2$ produced by flavoprotein enzyme systems. The systems studied in the present work, however, are of particular interest since they are independent of external sources of $H_2O_2$. How far such systems are of significance *in vivo* is not yet known. In some cases the reactions investigated proceed rapidly at physiological concentrations of $Mn^{2+}$, but the substrates oxidized in these cases are not known as intermediates in metabolism. However, only a few compounds have as yet been tested in the system. The requirements of the system for $Mn^{2+}$ in the oxidation of oxaloacetate are rather high in relation to normal physiological concentrations of $Mn^{2+}$. It therefore seems doubtful whether this oxidation is of significance *in vivo* except possibly under conditions of manganese toxicity particularly as the oxidation product, presumably malonic acid, is known to inhibit the tricarboxylic acid cycle.

## SUMMARY

1. The oxidation of oxalate, oxaloacetate, ketomalonate and dihydroxytartrate by oxygen is catalysed by peroxidase systems in presence of $Mn^{2+}$.

2. It is suggested that these oxidations and that of dihydroxymaleate in presence of peroxidase systems and $Mn^{2+}$ involves the oxidation of $Mn^{2+}$.

*Note.* In a paper, which appeared after the present work was submitted for publication, Chance (1952) put forward an explanation of the effect of $Mn^{2+}$ on the oxidation of dihydroxymaleic acid based on the activating effect of $Mn^{2+}$ ions on a peroxidase-hydrogen peroxide complex and not dependent on manganese oxidation. If this explanation is correct the manganese oxidation observed on the present work in the system peroxidase-dihydroxymaleic acid-$Mn^{2+}$ is a side reaction and dependent on the high concentration of $Mn^{2+}$ used. In view of the results obtained with other substrates, however, it seems reasonable to suggest that manganese oxidation is not a side reaction and that the absence of accumulated manganese oxidation products at low $Mn^{2+}$ concentration is due to their rapid reduction by the dihydroxymaleic acid.

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# Fermentation of Soluble Sugars by Anaerobic Holotrich Ciliate Protozoa of the Genera *Isotricha* and *Dasytricha*

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The glycolytic mechanisms of the obligatory anaerobic ciliates, which occur for example in great numbers in the rumens of sheep, oxen and goats, have never been adequately investigated. Hitherto the prevailing view has been that such Protozoa, even if seeming to ferment fodder carbohydrates, really do so by virtue of bacterial action in their body cavities (see, for example, van der Wath & Myburgh, 1941). It has recently been shown, however (Oxford, 1951; Masson & Oxford, 1951), that one important group of large rumen Protozoa, the holotrich ciliates, can very rapidly ferment glucose and certain other soluble hexoses, and oligo- and poly-saccharides, when the cells are separated from rumen contents and washed relatively free of extraneous bacteria. Such a fermentation will still take place even if the living Protozoa have been in contact with bactericidal concentrations of streptomycin for several days (Heald, Oxford & Sugden, 1952).

Since no evidence has been obtained by cultural studies that these holotrichs normally contain or ingest appreciable numbers of bacteria (Sugden & Oxford, 1952*a*, *b*), the inference seems clear that they must possess a powerful glycolytic mechanism of their own. This paper is mainly concerned with the action of washed, streptomycin-treated and starved sheep's rumen holotrichs on various soluble carbohydrates, together with a detailed study of the products of the fermentation of glucose by these Protozoa.

Some points of difference between these fermentation studies and a conventional bacterial fermentation must be mentioned at the outset in order that unprofitable comparisons between protozoan and bacterial action may be avoided:

(1) The protozoan suspensions used, even although virtually bacteria-free, were not a pure culture of one species only. There are three species of holotrichs present in the sheep's rumen, namely, *Isotricha prostoma*, *I. intestinalis* and *Dasytricha ruminantium*. No easy method of separation or cultivation of any one species has yet been devised (Sugden & Oxford, 1952*b*), nor can it be guaranteed that the same sheep on a standard hay diet will always yield a mixture of holotrichs containing the three species in constant relative proportions. This is probably of little importance in our fermentation study since microscopic examination has shown that all three species behave in the same way towards soluble fermentable sugars (Sugden & Oxford, 1952*b*).

(2) A very much greater proportion of the sugar disappearing from solution during the protozoan fermentation is stored in the cells as reserve starch-like polysaccharide than would be the case in an anaerobic bacterial glucose fermentation. This is mainly due to the size and internal structure of the Protozoa, e.g. the smallest of the three holotrichs, *D. ruminantium*, has dimensions of approximately $60 \times 40 \times 40\,\mu$. This is 100000 times as large as an average rumen coccus of diameter $1\,\mu$. The holotrich polysaccharide storage capacity is so great that the protein content of the fully loaded cell may be as little as 10% of the dry matter (Oxford, 1951).

(3) The ratio, carbon in cells to carbon in substrate at the beginning, is much greater than is usual for a bacterial fermentation. Changes in the carbon in the cells during fermentation have therefore to be taken into account in compiling balance sheets of the fermentation.

## METHODS

### *Material*

*Sheep as a source of holotrich ciliate Protozoa.* Rumen liquor was obtained from 3-year-old Cheviot wethers which were maintained exclusively on a virtually starch-free diet of hay fed at intervals of 12 hr. The sheep had been fitted with rumen cannulae by Dr A. T. Phillipson of this Institute (Phillipson & Innes, 1939).

*Preparation of virtually bacteria-free holotrich ciliates.* Samples of the rumen contents were taken with wide-bore glass tubes through the rumen cannulae 2 hr. after the sheep had been fed. At this point the storage of carbohydrate in the rumen micro-organisms of hay-fed sheep is at a maximum (Heald, 1951*a*). If samples were taken at a later period after feeding, poorer separation of the Protozoa was achieved. The samples were strained through six thicknesses of surgical gauze and were allowed to stand at 40° for 1–1·5 hr. During this period, the holotrich ciliates settled to the bottom together with much debris. The supernatant was decanted, the bottom layer was diluted slightly with acetate buffer (see below), and to this was then added glucose equivalent to 4–5 g./l. of original sample. The mixture was stirred, poured into separating funnels and allowed to stand

at 40° for 1–1·5 hr. The holotrich ciliates settled out as a thick white layer (Oxford, 1951) and were run off into sterile 50 ml. tubes containing acetate buffer. These were allowed to stand at 40°, when the gas produced by the ciliates caused any debris to rise to the top of the tubes. This was poured off, and the Protozoa were washed several times with the buffer by decantation. They were finally allowed to stand for 48 hr. in acetate buffer containing streptomycin (0·8 m-mole/l.), the buffer and streptomycin being changed at least twice and preferably three times during this period. In order to prevent excessive bursting of Protozoa, the washed cells from 1 l. of rumen contents were distributed between at least four 50 ml. tubes. Also for this reason, it was desirable that the buffer be changed frequently during the starvation period. After 48 hr. the Protozoa were sufficiently empty of storage polysaccharide to be used for further experiments. Microscopical examination then showed that all the living Protozoa were holotrich ciliates, and that all three species of rumen holotrichs were invariably present.

### *Composition of buffers*

*Acetate buffer for separation of the Protozoa from rumen contents.* This was based, as regards Na, on the composition of sheep saliva (McDougall, 1948), and contained NaCl, 5·0 g.; $CH_3COONa$, 1·5 g.; $K_2HPO_4$, 1·0 g.; $KH_2PO_4$, 0·3 g.; $MgSO_4.7H_2O$, 0·10 g.; distilled water, 1000 ml.; pH, 7·3–7·4.

*Phosphate-saline.* Since the Protozoa did not survive in 0·1 M or 0·2 M-sodium phosphate buffer alone, the buffer finally used had the following composition: NaCl, 5·0 g.; $K_2HPO_4$, 3·0 g.; $KH_2PO_4$, 1·0 g.; distilled water, 1000 ml.; pH, 7·3–7·4. This was used for fermentation studies and for some manometric experiments.

*Bicarbonate-saline.* This was the buffer, pH 7·4, gassed with 5 % $CO_2$ : 95 % $N_2$ (v/v) described by Krebs & Henseleit (1932). Although suitable for experiments of up to 2 hr. duration it was unsuitable after 4 hr., since many Protozoa burst.

### *Manometric methods*

Measurement of acid and gas production was carried out in standard Warburg flasks fitted with one or two side arms as required. In some experiments requiring two substrates, flasks with a single side arm were used, the second substrate being added from a dangling tube (Keilin, 1929).

Anaerobiosis was obtained by placing a stick of scraped yellow phosphorus in the centre well. $H_2$ production was measured in phosphate-saline by absorbing $CO_2$ in KOH contained in the centre well. At the end of the experiment 'bound' $CO_2$ was liberated by tipping in 0·2 ml. 10 N-$H_2SO_4$ from a second side arm.

### *Large-scale anaerobic fermentations*

*Method.* The fermentation liquid (50–100 ml.) was contained in a Büchner flask maintained at 40° in a water bath, and continuously swept by an $O_2$ and $CO_2$-free stream of $N_2$. The gas was delivered below the surface of the liquid. The outgoing gases were passed first through a tube of anhydrous magnesium perchlorate and then through two tared tubes of soda asbestos (British Drug Houses microanalytical reagent). The $CO_2$-free gases were mixed with dry $CO_2$-free $O_2$ and were combusted in a furnace over hot copper oxide, the products of combustion being absorbed in anhydrous magnesium perchlorate and soda asbestos contained in tared tubes. From the increase in the weight of these tubes the amount of $H_2$ and hydrocarbon gases (if any) could be determined.

*Inoculum.* This consisted of a suspension of the Protozoa prepared as described above. Usually the washed Protozoa from 4 to 6 l. of rumen liquor served as inoculum for 100 ml. medium, containing not more than 0·4 % (w/v) glucose. They were washed four or five times in the centrifuge with phosphate-saline at 40° and finally resuspended in buffer. A known volume was added to the flask, which was then swept with a slow continuous stream of $N_2$ for about 3 hr. and the fermentation was ended by the addition of 2 ml. of 10 N-$H_2SO_4$ to release bound $CO_2$. The flask was then swept out for a further hour. The contents were centrifuged and the Protozoa were made up to their original volume in fresh buffer. The samples were analysed as described below.

*Fermentation with [$^{14}$C]glucose.* The technique adopted was that outlined by Umbreit, Burris & Stauffer (1949) and used by Cochrane & Gibbs (1951) and Wiame & Doudoroff (1951). Protozoa were allowed to metabolize glucose to which randomly labelled [$^{14}$C]glucose (10 or 30 $\mu$c.) had been added, and were then washed and placed in Warburg manometers and allowed to ferment in the presence and absence of exogenous unlabelled glucose. The $CO_2$ evolved was collected in 10 N-NaOH in the centre well. After a suitable time the fermentation was stopped, by the acid-tip method, and the carbonate solution removed from the centre wells of the manometers. $Ba^{14}CO_3$ was precipitated in the presence of carrier carbonate from these solutions and counted at infinite thickness using an end-window counter.

### *Analytical methods*

*Streptomycin.* Streptomycin was estimated by the colorimetric method of Boxer, Jelinek & Leghorn (1947) as used for quantities of 200–1000 $\mu$g.

*Carbohydrates.* Glucose was estimated by the method of Hagedorn & Jensen (1923) by measuring the reducing value before and after fermentation of the solutions with Distiller's Company yeast (Mann, 1946).

Protozoan polysaccharide, after conversion to glucose by acid hydrolysis under the conditions described by Heald (1951 *a*), was estimated similarly.

*Total carbon.* The method of Heald (1951 *b*) was used with the following modification. Since the fermentation solutions contained large amounts of NaCl, it was found necessary to remove all $Cl_2$ formed on oxidation by absorption in $H_2O_2$ (100 vol.), contained in a spiral bubbler.

*Volatile acids.* These were distilled in the Markham (1942) still. The acids were separated and identified by means of gas-liquid partition chromatography (James & Martin, 1952).

*Lactic acid.* This was determined by the method of Friedemann & Graeser (1933) after removal of glucose by copper-lime (Van Slyke, 1917).

*Succinic acid.* The method was that of Krebs (1937) using sheep-heart succinoxidase.

*Non-volatile acids other than lactic acid.* The method used was that of Birkinshaw & Raistrick (1931), after first concentrating the metabolism solution, under reduced pressure, to a small volume.

*Total nitrogen.* This was estimated by the method of Chibnall, Rees & Williams (1943).

*Ammonia and urea.* The method of Conway (1947) was used.

*Volatile neutral and non-volatile neutral compounds.* These were determined as described by Neish (1946).

*Substrates, etc.* Streptomycin was used as the $CaCl_2$ complex supplied by Glaxo Laboratories Ltd. The carbohydrates were good commercial samples (L. Light or Kerfoot, Biochemical Reagent quality) with the exception of dahlia inulin and rye-grass fructosan, which were supplied by the late Dr E. G. V. Percival and Dr P. C. Arni (of this Institute) respectively.

[$^{14}$C]*Glucose.* This was supplied by the Radiochemical Centre, Amersham, Bucks.

*Units.* Since the gas evolved during fermentation may contain $H_2$ as well as $CO_2$, results in Fig. 1 *a* and *b* are given as '$\mu$l. gas' produced in bicarbonate saline/0·1 mg. protozoan N. These values were obtained by multiplying mm. pressure change by the appropriate manometer constant for $CO_2$ ($k_{CO_2}$). It is recognized that these figures have no absolute meaning but may be used to show the relative rate of gas production by the Protozoa under the experimental conditions.

## RESULTS

### *Effect of streptomycin on the holotrich ciliates*

Results with added pure cultures of bacteria have been summarized elsewhere (Heald *et al.* 1952). They showed that a concentration of 0·8 m-mole/l. of streptomycin base (500–600 $\mu$g./ml.) was effective in completely suppressing all bacteria, added or otherwise. Furthermore, no bacterial colonies developed if the streaked test plates were incubated anaerobically in a 95 % $N_2$:5 % $CO_2$ atmosphere. It was therefore concluded that streptomycin at this concentration had rendered the living Protozoa virtually bacteria-free, and that no substantial interference by bacterial action in any fermentation study restricted to 3 hr. or less need be feared.

The concentration of streptomycin used was many times the usual lethal dose for bacteria (cf. Pulvertaft, 1952). It had no visible effect on the holotrichs in 2–3 days, i.e. neither on their motility nor on the gradual diminution of their polysaccharide reserves. The suppression of bacteria was not due to a dilution effect caused by replacing the buffer, since there was good bacterial growth on plates inoculated from non-streptomycin-treated control cultures containing similarly washed ciliates. The Protozoa did not decompose the antibiotic, since a quantitative recovery of it was obtained after a 24 hr. treatment of the cells with streptomycin (0·8 m-mole/l.).

### *Manometric experiments with various substrates in bicarbonate buffer*

Since the holotrich ciliates stored large quantities of polysaccharide, part of which was still present after 48 hr. of starvation, it was to be expected that the endogenous fermentation would then still be appreciable. For this reason, and because the protozoan suspensions contained three separate species, the relative proportions of which might vary from time to time, it is clear that no simple basis exists for the quantitative comparison of manometric results obtained with different suspensions, even if all were derived from the same sheep. Nevertheless, the fermentation of glucose was strikingly evident. Fructose, sucrose, raffinose, inulin and rye-grass levan were fermented at rates comparable to glucose; cellobiose at a much slower rate; and xylose, galactose, mannose, turanose, maltose, lactose, melibiose, melezitose, soluble starch (as paste) and sodium glucuronate, not at all.

### *Effect of streptomycin on the rate of acid production from glucose*

When the activity of washed and starved cells which had been in contact with streptomycin was compared with a suspension not treated with streptomycin, there was no essential difference in the rate of acid production (Fig. 1*b*). Similarly, the addition of streptomycin during the metabolism of glucose by washed streptomycin-treated cells, did not produce a measurable effect (Fig. 1*a*).

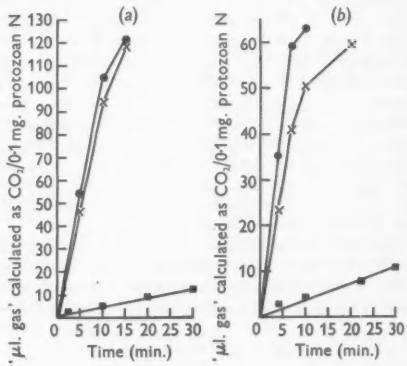


Fig. 1. (*a*) The effect of streptomycin on the rate of fermentation of glucose by Protozoa previously treated with streptomycin. Each flask contained 292 $\mu$g. cell N in 2 ml. of bicarbonate-saline. Side arms contained 0·5 ml. of 0·01 M-glucose. ●—●, glucose; ×—×, glucose + streptomycin (1·5 mg.) in buffer (0·2 ml.); ■—■, endogenous substrate.

(*b*) Comparison of the rate of fermentation of glucose by two protozoan suspensions, one treated with streptomycin and one untreated. Each flask contained the cells in 2 ml. of buffer. Side arms contained 0·5 ml. of 0·01 M-substrate. ●—●, cells treated with streptomycin; protozoan N = 336 $\mu$g./manometer. ×—×, cells not treated with streptomycin; protozoan N = 414 $\mu$g./manometer.

In both experiments the gas phase was 95 % $N_2$:5 % $CO_2$; temp., 40°. Anaerobiosis was obtained by placing a stick of yellow phosphorus in the centre well. For method of calculation of '$\mu$l. gas' see text.

Table 1. *The production of hydrogen and carbon dioxide during the fermentation of glucose by holotrich ciliates of the sheep's rumen*

(Each manometer contained 2 ml. of Protozoa in phosphate saline, pH 7·4; gas, $N_2$; temp., 40°. Glucose added in 0·5 ml. buffer.)

| Protozoan suspension no. | Glucose added (μmoles) | Time (min.) | Total gas produced (μl.) | Hydrogen produced (μl.) | Carbon dioxide produced (μl.) | Ratio $\frac{\text{carbon dioxide}}{\text{hydrogen}}$ |
|---|---|---|---|---|---|---|
| 1 | 5 | 10 | 175 | 90 | 85 | 0·95 |
| 2 | 5 | 12 | 96 | 51 | 45 | 0·89 |
| 3 | 5 | 18 | 272 | 130 | 142 | 1·09 |
| 4 | 100 | 30 | 190 | 107 | 83 | 0·77 |
| | | 60 | 290 | 162 | 128 | 0·79 |
| | | 90 | 324 | 149 | 175 | 1·13 |
| 5 | 100 | 60 | 103 | 55 | 48 | 0·87 |
| | | 120 | 196 | 104 | 92 | 0·88 |
| | | 180 | 158 | 75 | 83 | 1·12 |

*Manometric studies with glucose in a bicarbonate-free buffer*

In order to obtain a measure of true fermentation carbon dioxide and of any other gases evolved, manometric experiments were carried out in the phosphate buffer. Table 1 shows that a gas other than carbon dioxide was in fact obtained under these conditions, and this was later shown, by combustion over copper oxide, always to be entirely hydrogen with no admixture of methane. It appeared probable that hydrogen was reabsorbed during the later stages of the fermentation of a large excess of glucose.

*Effect of oxygen on glucose fermentation*

Suspensions of Protozoa, when shaken in air, soon lost all power of ciliary motion, and burst. Under these conditions, although still feebly alive for a period, it was found that hardly any glucose was used and that the uptake of oxygen was very small indeed.

*Large-scale fermentation of glucose by holotrich ciliates*

*Fermentation in phosphate buffer.* No fermentation was allowed to proceed beyond 3 hr. The only detectable fermentation products were lactic, acetic, butyric and sometimes traces of propionic acids together with carbon dioxide and hydrogen. Volatile fatty acids higher than butyric were not produced. Volatile and non-volatile neutral and non-volatile acidic products (including succinic acid) seemed to be present, if at all, in the merest traces. No formic acid was detected. The results of six fermentations are summarized in Table 2. It will be noted that more than half of the glucose fermented is accounted for by an increase of glucose-containing cellular material, presumably storage glucosan for the most part (cf. Masson & Oxford (1951) who attempted with only moderate success to isolate this starch-like material quantitatively by perchloric acid extraction). The carbon recoveries and the oxidation-reduction index indicated that the bulk of the glucose fermented seemed to be accounted for satisfactorily. This calculation assumes, however, that no loss of cellular material, other than storage glucosan, took place during fermentation. Since lactic and acetic acids, with oxidation-reduction values identical with that of glucose, might conceivably also arise from lower amino-acids by deamination, it was considered advisable to prepare a carbon and nitrogen balance sheet for the whole fermentation system. The results for four glucose fermentations are summarized in Table 3. It will be noted that there was always a considerable loss of cellular nitrogen. Since the fermentation liquid gave no reactions for ammonium salts even when the fermentation was conducted in a closed system (Table 3, Exp. 6), nor for amino-acids (by ninhydrin test) or urea, it was assumed that the lost nitrogen was probably present in solution as soluble protein or polypeptide, derived by breakdown of cellular protein. Hence carbon in organic nitrogenous compounds in solution might reasonably be arrived at by multiplying the weight loss of nitrogen from cells by three. Owing, presumably, to difficulties of sampling on so small a scale, these two independent values for soluble carbon as 'polypeptide' did not always exactly agree. Even so, there was usually still some carbon unaccounted for in solution, the origin of which is obscure. It is conceivable that this is the sum of numerous products arising from cellular autolysis, but individually present in traces only, beyond the limit of detection by the methods used.

The extent of the loss of cellular substance, other than storage polysaccharide during fermentation, was also determined by another method based on a comparison of the extra carbon stored in the cells as reserve glucosan (and liberated as glucose on hydrolysis) with the total increase in cellular carbon

Table 2. *The products of the fermentation of glucose by rumen holotrich ciliates, in phosphate-saline*

(Glucose (100 ml. 0·4 %) fermented in $N_2$ stream at 40°; initial pH, 7·4; CO absorbed in soda asbestos; $H_2$ determined by combustion.)

| Exp. | Glucose: Fermented (m-mole) | Glucose: Stored as glucosan (m-mole) | Lactic acid (m-mole) | Acetic acid (m-mole) | Propionic acid (m-mole) | Butyric acid (m-mole) | Carbon dioxide (m-mole) | Hydrogen (m-mole) | Apparent carbon recovery (%) | Hydrogen/oxygen index for fermentation products |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0·96 | 0·528 | 0·21 | 0·128 | 0 | 0·128 | 0·44 | 0·58* | 88·4 | 2·01 |
| 2 | 1·01 | 0·568 | 0·31 | 0 | 0 | 0·22 | 0·37 | 0·49* | 94·6 | 2·13 |
| 3 | 1·86 | 1·18 | 0·53 | 0·23 | 0·056 | 0·142 | 0·48 | 0·61 | 94·0 | 1·99 |
| 4 | 2·16 | 1·26 | 0·54 | 0·29 | 0·08 | 0·30 | 0·76 | 1·05 | 94·3 | 2·08 |
| 5 | 1·83 | 1·42 | 0·33 | 0·25 | 0 | 0·25 | 0·56 | 0·75* | 104·5 | 2·01 |
| 6 | 0·338 | — | 0·086 | 0·065 | 0 | 0·065 | 0·122 | 0·16* | — | 2·13 |

* Calculated on the basis that carbon dioxide/hydrogen = 0·75.

Table 3. *Overall fermentation balance sheet for holotrich ciliate fermentation of glucose, attention being paid to changes in cellular carbon and nitrogen and also storage polysaccharide*

(For details see Table 2.)

| Ref. no. of Exp. (see Table 2) | Carbon in glucose used up from solution (mg.) $G$ | Carbon in carbon dioxide evolved (mg.) $M$ (see Table 2) | Carbon in cells: Before fermentation (mg.) $A$ | Carbon in cells: After fermentation (mg.) $X$ | Increase in total protozoan carbon during fermentation (mg.) $(X-A)$ | Carbon in solution at end, other than glucose carbon (mg.) $S$ | Total of carbon accounted for (%) $\frac{(X-A)+S+M}{G}\times 100$ |
|---|---|---|---|---|---|---|---|
| 3 | 134·2 | 5·4 | 63·6 | 123·6 | 60·0 | 74·4 | 104 |
| 4 | 143·3 | 9·1 | 130·8 | 180·0 | 49·2 | 80·0 | 97 |
| 5 | 131·4 | 6·7 | 103·2 | 185·4 | 82·2 | 36·8 | 96·5 |
| 6 (closed system) | 26·2 | 1·4 | 27·8 | 34·9 | — | 13·4 | 83·5 |

| Ref. no. of Exp. (see Table 2) | Carbon in form of protozoan glucosan (mg.): Before fermentation | Carbon in form of protozoan glucosan (mg.): After fermentation | Increase in glucosan carbon during fermentation (mg.) $P$ | Net decrease in carbon in protozoan cellular substance other than polysaccharide (mg.) $P-(X-A)$ | Nitrogen in cells: Before fermentation (mg.) | Nitrogen in cells: After fermentation (mg.) | Nitrogen in solution after fermentation (mg.) |
|---|---|---|---|---|---|---|---|
| 3 | 43·4 | 129·1 | 85·7 | 25·7 | 11·0 | 6·0 | 3·2 |
| 4 | 96·2 | 187·2 | 91·0 | 41·8 | 16·9 | 13·7 | 4·0 |
| 5 | 52·8 | 155·5 | 102·7 | 20·5 | 14·4 | 10·9 | — |
| 6 (closed system) | — | — | — | — | 3·9 | 2·6 | 0·8 |

Table 4. *Endogenous fermentation balance sheet for holotrich ciliate Protozoa in phosphate buffer*

(Each experiment with approx. 100 ml. buffer pH 7·3, ran for approx. 3 hr. in $N_2$ stream at 40°. $CO_2$ absorbed in soda asbestos.)

| Initial state of Protozoa | Carbon in carbon dioxide evolved (mg.) | Total carbon in cells: Before fermentation (mg.) | Total carbon in cells: After fermentation (mg.) | Carbon in protozoan glucosan: Before fermentation (mg.) | Carbon in protozoan glucosan: After fermentation (mg.) | Carbon in solution at end (mg.) | Carbon as soluble acidic products of fermentation (mg.): Lactic | Acetic | Butyric | Nitrogen in solution after fermentation (mg.) |
|---|---|---|---|---|---|---|---|---|---|---|
| After 2 days starvation | 0·24 | 27·8 | 24·9 | — | — | 3·6 | 0·6 | 0·65 | 0·65 | 1·0 |
| Immediately after glucose fermentation | 0·96* | 61·2 | 46·8 | 62† | 34·8 | 15·6 | 3·4 | 1·1 | 0·95 | 2·7 |

* Gaseous carbon dioxide/hydrogen ratio was 0·6.

† Probably a little too high. The cells nevertheless were extremely well-filled with storage polysaccharide, when examined microscopically after staining with Lugol's iodine.

as determined by wet combustion (see also Table 3). The net decrease in the carbon of non-glucosan cellular substance was rather greater than the apparent production of soluble 'polypeptide'. This is to be expected since some carbon dioxide and a little of the acidic fermentation products have probably arisen from original cellular substance during the fermentation. The possible magnitude of this contribution was indicated by a no-substrate control fermentation (Table 4). As might be anticipated, the soluble products of endogenous fermentation (particularly lactic acid) were relatively much greater in amount when well-filled Protozoa, taken immediately after a glucose fermentation, were used. Even the starved Protozoa, however, still produced acetic and butyric acids as well as 'polypeptide'. It is safe to conclude, therefore, that the endogenous fermentation of glucosan is always qualitatively similar to the fermentation of added glucose.

*Fermentation in bicarbonate buffer.* An experiment was carried out to determine whether a reasonably high concentration of bicarbonate, such as exists in the rumen, affected the end products from the fermentation of glucose. When a fermentation was carried out in a modified bicarbonate saline containing $3{\cdot}55 \times 10^{-2}$ M-$HCO_3^-$ (pH approx. 6·5) under a 100% carbon dioxide atmosphere, the percentages of the carbon fermented which appeared as lactic, acetic, propionic and butyric acids were 12·9, 4·2, 0·45 and 6·5, respectively. These values did not differ sufficiently markedly from those found in Table 2 (fermentation in phosphate-saline in 100% nitrogen atmosphere) to suggest that a higher bicarbonate concentration had any effect on the fermentation. The pH chosen was within the normal range for actively fermenting rumen contents *in vivo*.

Table 5. *Effect of protozoan fermentation of added glucose on endogenous fermentation of* [$^{14}$C]*-storage polysaccharide*

(Two experiments (suspensions (*a*) and (*b*)) carried out in phosphate-saline buffer at pH 7·2. The experiments were carried out in Warburg manometers. Each flask contained 2 ml. of the protozoan suspension. The side arms contained either 200 $\mu$moles glucose in 0·5 ml. of buffer, or 0·5 ml. of buffer, as required. The $CO_2$ evolved was absorbed in KOH (10%, w/v) in the centre well; gas phase, 100% $N_2$; temp., 40°. Radioactivity measured at infinite thickness to an accuracy of ±5%.)

| Substrate | Carbon dioxide produced ($\mu$l.) | Activity in carbon dioxide counted as $BaCO_3$ (counts/min.) |
|---|---|---|
| Endogenous | (*a*) 133 | 141 |
| | (*b*) 168 | 101 |
| Glucose | (*a*) 586 | 52 |
| | (*b*) 736 | 35 |

*The effect of glucose fermentation on the endogenous fermentation of storage polysaccharide*

In view of the large endogenous fermentation taking place in the absence of added carbohydrate, it was of interest to determine whether this fermentation was suppressed during the fermentation of glucose. A fermentation, first of labelled, and then of unlabelled glucose by the same cells was therefore carried out (see Methods section). The results are shown in Table 5. Taking into account the inevitable dilution of radioactive storage polysaccharide resulting from 'starch' formation in the subsequent fermentation of unlabelled glucose, it is considered that there was no extensive suppression of the endogenous fermentation in this experiment.

## DISCUSSION

If it be allowed that streptomycin, as our results seem to show, has no appreciable effect on the protozoan enzymes, it is a remarkable fact that the only soluble sugars from which the bacteria-free holotrich ciliates produce acid rapidly are those liable to occur in the sheep's fodder, namely, glucose, fructose, sucrose, raffinose, inulin and grass fructosan. These Protozoa have also a certain limited power of fermenting cellobiose, and presumably other $\beta$-glucosides of true plant origin. In the main these results with washed suspensions are in harmony with those of Sugden & Oxford (1952*b*) on the effect of soluble sugars in prolonging life of the Protozoa beyond 2 days. It is clear that a number of sugars, like maltose, and the pentoses, which are known to be readily fermented by mixed rumen bacteria, are of little use to the rumen holotrich ciliates. Nevertheless, the protozoan fermentation products from glucose, in the absence of bacteria, are qualitatively just those which would arise from glucose *in vitro* by the action of a mixed population of rumen bacteria, namely, lactic, acetic, propionic and butyric acids, carbon dioxide and hydrogen (cf. Elsden, 1945). The relative proportion of propionic acid is, however, much smaller in the protozoan fermentation, and the presence or absence of bicarbonate does not appear to affect the yields of the respective acids. It is doubtful, therefore, whether carbon dioxide absorption plays an important part in the metabolism of these Protozoa, even though in their natural habitat, the rumen, they constantly live in presence of a high bicarbonate concentration.

As regards the host animal, the significance of the rapid rate of glucosan storage by these Protozoa is twofold: (*a*) the rumen bacteria are prevented from fermenting all soluble carbohydrates at a rapid rate: (*b*) when no soluble fodder carbohydrate remains, the endogenous fermentation of the protozoan

storage glucosan releases into the rumen a steady flow of lactic, acetic and butyric acids. The first-named acid is probably converted into volatile fatty acids, chiefly propionic, by known bacterial mechanisms (cf. Elsden, 1945; Johns, 1951).

It may be pointed out that this is the first time that rumen ciliate Protozoa have been shown to possess a vigorous carbohydrate metabolism in absence of bacteria. Furthermore, it is clear that, in this respect at least, the ciliates are beneficial rather than harmful to the host animal.

## SUMMARY

1. Suspensions of virtually bacteria-free and starved holotrich ciliates separated from sheep's rumen contents, containing *Isotricha prostoma*, *I. intestinalis* and *Dasytricha ruminantium*, produced acids and gas at a rapid rate under anaerobic conditions from the following soluble carbohydrates: glucose, fructose, sucrose, raffinose, inulin, rye-grass levan and, to a much lesser degree, cellobiose. No other soluble carbohydrate was attacked. Glucose was not attacked in presence of oxygen.

2. The relatively high concentration of streptomycin used to kill bacteria associated with the Protozoa had little effect upon the viability or fermentative activity of the latter.

3. The fermentation products from glucose were lactic, acetic, propionic (traces) and butyric acids, carbon dioxide and hydrogen. Soluble nitrogenous compounds, which were not ammonia, urea or amino-acids, were also produced.

4. In the first 3 hr. the starved ciliates stored more glucose as polysaccharide than was fermented to acids.

5. The endogenous fermentative activity of the ciliates containing storage glucosan yielded lactic, acetic and butyric acids.

6. Glucose fermentation did not extensively suppress the endogenous fermentation of storage polysaccharide.

7. The use of carbon dioxide instead of nitrogen in the gas phase had little apparent effect upon the protozoan glucose fermentation.

8. The importance of these ciliates as fermentative agents in the rumen is discussed.

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